

DIETARY RED PALM OIL-SUPPLEMENTATION OFFERS CARDIOPROTECTION AGAINST ISCHAEMIA/REPERFUSION INJURY: POSSIBLE CELLULAR MECHANISMS INVOLVED

by

Adriaan Johannes Esterhuyse

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Promoter: Dr Jacques van Rooyen
Department of Physiological Sciences
University of Stellenbosch

Co-promoter: Dr Eugene F du Toit
Department of Medical Physiology and Biochemistry
University of Stellenbosch

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LIST OF ABBREVIATIONS

α	Alpha
AA	Arachidonic acid
ALA	α -Linolenic acid
AO	Aortic output
BAD	Pro-apoptotic protein BAD
β	Beta
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CF	Coronary flow
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
Chol	Cholesterol
Chol/RPO	Cholesterol/Red Palm Oil
CoQ ₁₀	Coenzyme Q ₁₀
COX-2	Cyclooxygenase-2
CVD	Cardiovascular disease
DAF-2/DA	Diaminofluorescein-2/diacetate
DHA	Docosahexaenoic acid
Δ	Delta
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated protein kinase
γ	Gamma

H ₂ O ₂	Hydrogen peroxide
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HR	Heart rate
JNK	c-JunN-terminal kinase
LA	Linoleic acid
LDL	Low density lipoprotein
LPO	Lipid hydroperoxide
LTs	Leukotrienes
LVDevP	Left ventricular developed pressure
LVDP	Left ventricular diastolic pressure
LVSP	Left ventricular systolic pressure
MAPKs	Mitogen-activated protein kinases
MKK6	MAPK6
MUFAs	Monounsaturated fatty acids
NO	Nitric oxide
NOS	Nitric oxide synthase
O ₂ ⁻	Superoxide
OH	Hydroxyl radical
ONOO ⁻	Peroxynitrite
P/S	Polyunsaturated/saturated ratio
p38	p38 Mitogen-activated protein kinase
PARP	Poly(ADP-ribose)polymerase
PDK	Phosphoinositide-dependent protein kinase
PGIs	Prostacyclines
PGs	Prostaglandins

PKB/Akt	Serine/threonine protein kinase, Protein kinase B or AKT
PKC	Protein kinase C
PPM	Parts per million
PUFAs	Polyunsaturated fatty acids
RBDPO	Bleached and deodorized palm oil
RC	Rat chow
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPO	Red Palm Oil
RPP	Rate pressure product
SF	Saturated animal fat
SFAs	Saturated fatty acids
SOD	Superoxide dismutase
SRC	Standard rat chow
SSO	Sunflower seed oil
TAG	Triglycerides
TC	Total cholesterol
Thr-X-Tyr	Threonine-X-Tyrosine
TLC	Thin layer chromatography
TNF α	Tumour necrosis factor alpha
TX	Thromboxanes
TXA ₂	Thromboxane A ₂
U/S	Unsaturated/saturated ratio
WHO	World Health Organization

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:.....

Date:

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ABSTRACT

Activation of the NO-cGMP pathway is associated with myocardial protection against ischaemia/reperfusion injury. However, high-cholesterol diets alter function of this pathway and these alterations have been implicated in both ischaemic/reperfusion injury and the development of ischaemic heart disease. Little is known about the effects of supplements such as Red Palm Oil (RPO) on the myocardial NO-cGMP-signalling pathway. RPO consists of saturated, mono-unsaturated and poly-unsaturated fatty acids and is rich in antioxidants such as β -carotene and Vitamin E (tocopherols and tocotrienols). The aims of this study were: 1) to determine whether dietary RPO-supplementation protects against ischaemia/reperfusion injury in rats fed a standard rat chow (control) and cholesterol-enriched diets and 2) if so, to investigate possible mechanisms for this protection.

Male Long-Evans rats were fed a standard rat chow or a standard rat chow plus cholesterol and/or RPO-supplementation for 6 weeks. Myocardial functional recovery was measured and hearts were freeze-clamped for determination of myocardial phospholipid, cAMP/cGMP concentrations, total myocardial nitric oxide concentrations, lipid hydroperoxide production and superoxide dismutase- and nitric oxide synthase activity in isolated rat hearts subjected to 25 minutes of normothermic total global ischaemia. In addition, the degree of phosphorylation of extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal protein kinase (JNK) and protein kinase B (PKB/Akt) was investigated. Furthermore, the effect of RPO-supplementation on caspase-3 activation and poly (ADP-ribose) polymerase (PARP)-cleavage in hearts subjected to ischaemia and reperfusion was also investigated.

Our data show that dietary RPO-supplementation protects the hearts of rats on a standard rat chow (control) and hypercholesterolaemic diet against ischaemia/reperfusion injury as reflected by improved aortic output recovery. Increased intracellular cardiomyocyte NO concentrations as observed in control hearts supplemented with RPO after 120 minutes hypoxia may contribute to the elevated cGMP concentration and may confer some of the cardioprotection to the ischaemic/reperfused heart. Although improved functional recovery with RPO-supplementation of a high-cholesterol diet was also associated with an increase in intracellular cardiomyocyte NO production after hypoxia compared to the non-hypoxic conditions, it could not be linked to increased NO-cGMP signalling. These data are in agreement with other studies, which showed that high-cholesterol diet impairs NO-cGMP signalling and confirms our hypothesis that elevated cGMP concentrations may not be the only mechanism of protection. We have also shown that RPO-supplementation caused increased phosphorylation of p38 and PKB, reduced phosphorylation of JNK and attenuation of PARP cleavage, which may contribute to the protection of the cell against apoptosis.

Based on our results we propose that the myocardial protection offered by RPO-supplementation of rats on a normal and hypercholesterolaemic diet may be associated with either its antioxidant characteristics and/or changes in the fatty acid composition of the myocardium during ischaemia/reperfusion. Furthermore, we demonstrated for the first time that RPO-supplementation protects the isolated perfused working rat heart during reperfusion from ischaemia/reperfusion-induced injury through a MAPK-dependent pathway.

UITTREKSEL

Aktivering van die NO-cGMP sein transduksie pad word geassosieer met miokardiale beskerming teen isgemie/herperfusie skade. Hoë cholesterol diëte verander egter die funksie van die pad en hierdie veranderings speel 'n rol in beide isgemie/herperfusie besering en die ontwikkeling van isgemiese hartsiekte.

Daar is egter min inligting beskikbaar oor die uitwerking van aanvullings soos rooi palm olie (RPO) op die miokardiale NO-cGMP sein transduksie pad. RPO bevat versadigde, mono-onversadigde en poli-onversadigde vetsure en is ryk aan anti-oksidente nl. β -karotene en vitamien E (tokoferole en tokotriënole). Die doelwitte van hierdie studie was: 1) om vas te stel of 'n RPO-aanvulling beskerming bied teen isgemie/herperfusie besering in rotte wat gevoed is met 'n standaard rotmengsel (kontrole) en cholesterol-verrykte dieet en 2) indien wel, om moontlike meganismes van beskerming te ondersoek.

Long-Evans manlike rotte is vir 6 weke gevoer met 'n standaard rotmengsel of 'n standaard rotmengsel plus cholesterol en/of RPO-aanvulling. Miokardiale funksionele herstel is gemeet en harte is gevriesklamp vir die bepaling van miokardiale fosfolipied, cAMP/cGMP, totale stikstofoksied, lipied hidroperoksied, superoksied dismutase en stikstofoksied sintase in geïsoleerde rotharte wat vir 25 minute onderwerp was aan normotermiese totale globale isgemie. Hiermee saam is die graad van fosforilering van ekstrasellulêre sein gereguleerde kinase (ERK), p38 mitogeen-geaktiveerde proteïen kinase (p38 MAPK), c-Jun-N-terminale proteïenkinase (JNK) en

proteïen kinase B (PKB/Akt) ondersoek, asook kaspase-3 aktivering en poli (ADP-ribose) polimerase (PARP) kliewing in harte blootgestel aan isgemie en herperfusie.

Ons resultate toon dat RPO-aanvulling van rotte op 'n normale en hipercholesterolemiese dieet die hart beskerm soos getoon deur verbeterde herstel van aortiese uitset. Verhoogde intrasellulêre miokardiale NO vlakke in kontrole harte met 'n RPO-aanvulling wat blootgestel was aan 120 minute hipoksie, mag bygedra het tot die verhoogde cGMP vlakke en beskerming van die hart tydens isgemie en herperfusie. Alhoewel verbeterde funksionele herstel met RPO-aanvulling van 'n hoë cholesterol dieet ook geassosieer is met 'n toename in intrasellulêre miokardiale NO produksie ná hipoksiese toestande, kon dit nie verbind word met verhoogde aktivering van die NO-cGMP sein transduksie pad nie. Hierdie resultate stem ooreen met ander studies wat aangetoon het dat hoë-cholesterol diëte die NO-cGMP seipad onderdruk. Hierdie bevinding bevestig ons hipotese dat verhoogde cGMP vlakke moontlik nie die enigste beskermingsmeganisme is nie. Ons resultate het ook gewys dat RPO-aanvulling fosforilering van p38 en PKB/Akt verhoog, fosforilering van JNK verminder en PARP kliewing onderdruk. Dit dui op beskerming van die sel teen apoptose.

Ons resultate dui aan dat die miokardiale beskerming wat RPO-dieet aanvulling bied moontlik geassosieer kan word met sy anti-oksident eienskap en/of veranderinge in die vetsuur samestelling van die miokardium tydens isgemie/herperfusie. Ons het ook vir die eerste keer bewys dat RPO-aanvulling

die geïsoleerde geperfuseerde werkende rothart gedurende herperfusie beskerm teen isemie/herperfusie besering deur die aktivering en/of deaktivering van die MAPK afhanklike pad.

CHAPTER 1

INTRODUCTION

Palm oil and its liquid fraction, palm olein, are consumed worldwide as cooking oils and as constituents of margarines. These oils are also incorporated into fat blends used in the manufacturing of a variety of food products and in home food preparation. It plays a meaningful role in meeting energy needs and contributes to essential fatty acid (C18:2n-6) needs in many regions of the world (reviewed by Cottrell, 1991). Refined red palm oil (RPO) used in this study consists of 51% saturated fatty acids (SFAs), 38% monounsaturated fatty acids (MUFAs), 11% polyunsaturated fatty acids (PUFAs) and contains no less than 500 parts per million (ppm) carotenoids, 60% as β -carotene and 25% as α -carotene. The vitamin E content is about 500 ppm of which 70% is tocotrienols and 30% tocopherols (Nagendran *et al.*, 2000; Sundram *et al.*, 2003).

Most previous studies with palm oil in humans and animals have focused on lipoproteins and cardiovascular disease where clinical trials have evaluated the effect of palm oil on blood lipids and lipoproteins. These studies suggest that palm oil does not raise serum total cholesterol (TC) or LDL cholesterol levels to the extent expected when a moderate-fat, moderate-cholesterol diet is consumed. This can possibly be ascribed to the fatty acid composition of palm oil (Chandrasekharan, 1999; Theriault *et al.*, 1999; Kritchevsky, 2000; Sundram and Basiron). However, when hypercholesterolaemic subjects and high fat liquid formula diets were used, palm oil-supplementation appeared to raise TC and LDL cholesterol (Sundram and Basiron).

The use of RPO *per se* in previous studies has been limited and more literature refers to the use of fractionated tocotrienol in studies that predominantly focus on lipid metabolism, atherosclerosis and cancer. We therefore used dietary RPO that contained both fatty acids and antioxidants while we investigated the cellular mechanisms that may be involved in the cardioprotection against ischaemia/reperfusion injury.

To our knowledge Serbinova and co-workers (1992) were the first to demonstrate that a palm oil vitamin E mixture containing both α -tocopherol and α -tocotrienol improved reperfusion functional recovery in a Langendorff-perfused rat heart. This protection was due to the ability of both α -tocopherols and α -tocotrienols to scavenge free radicals during reperfusion, but the researchers argued that α -tocotrienols might have been the more potent free radical scavenger of the two.

The composition of RPO allows for this protection to be offered by either the fatty acid constituents or the anti-oxidative carotenoids, tocopherols and tocotrienols or both. Since the antioxidant content of RPO is very high, it may well be argued that protection may also be offered via the NO-cGMP pathway, but the possible role of the fatty acids also needs consideration and clarification. It has been shown that longer chain n-3 fatty acids offer protection against ischaemia/reperfusion injury through MAPK-, PKB/Akt- and caspase dependant pathways (Engelbrecht *et al.*, submitted).

To our knowledge the cardioprotection offered by RPO and the role of NO-cGMP signalling has not been investigated under normal and/or conditions such as ischaemia or reperfusion. Recent studies suggested that the MAPK, PKB/Akt and signal transduction caspases might also be involved in the regulation of apoptosis in response to myocardial ischaemia/reperfusion. Therefore, we used the ischaemic/reperfused working rat heart model to elucidate whether the function and activity of these pathways (NO-cGMP, MAPK, PKB/Akt and caspases) are altered and contribute to the red palm oil-induced protection against ischaemia/reperfusion injury.

Nitric Oxide (NO) is, by virtue of its vasodilator, antioxidant, anti-platelet and anti-neutrophil actions, an essential molecule for normal heart function (reviewed by Ferdinandy and Schultz, 2003). It has been shown to be cardioprotective in the ischaemic heart (reviewed by Bolli, 2001). However, NO is detrimental when it is combined with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), which rapidly decomposes to highly reactive oxidant species, leading to tissue injury (reviewed by Ferdinandy and Schultz, 2003). There is a critical balance between cellular concentrations of NO, O_2^- and superoxide dismutase (SOD) which physiologically favour NO production, but in pathological conditions such as ischaemia and reperfusion injury, result in $ONOO^-$ formation (reviewed by Ferdinandy and Schultz, 2003). A role for reactive oxygen species, including the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) have long been implicated in the pathogenesis of ischaemia/reperfusion injury. These oxygen free radicals can react with nucleic

acids, proteins and lipids, resulting in damage to the cell membrane or intracellular organelles (Gilham *et al.*, 1997).

Myocardial NO formation is increased during ischaemia and reperfusion, offering protection against ischaemia/reperfusion injury (Williams *et al.*, 1995; Araki *et al.*, 2000; Bolli, 2001). Protective effects of NO are mediated through the production of cGMP. NO is known to increase myocardial cGMP, and it has been suggested that the protective effect of NO is related to a mechanism secondary to the stimulation of guanylyl cyclase within the vascular wall or in ventricular myocytes (Beresewics *et al.*, 1995; Maulik *et al.*, 1995; Depré *et al.*, 1996). NO donors given during ischaemia possibly protect the myocardium by increasing tissue cGMP and decreasing cytosolic Ca^{2+} overload (Du Toit *et al.*, 2001). These investigators found that nitric oxide donor treatment reduces ischaemia/reperfusion injury by increasing cGMP concentrations and suggested that the cAMP-to-cGMP ratio might play an important role in NO induced cardioprotection. Maulik and co-workers (1995) showed that NO plays a significant role in transmembrane signalling in the ischaemic myocardium. This group suggested that NO signalling is switched off due to inactivation of NO by reactive oxygen species and was the first to suggest that reactive oxygen species may alter NO-cGMP signalling.

Increases in cAMP concentrations associated with ischaemia would increase Ca^{2+} concentrations and exacerbate ischaemic/reperfusion injury (Du Toit *et al.*, 2001). In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP induced increase in the slow inward calcium

current, thus leading to a decrease in cytosolic calcium levels (Summi and Sperelakis, 1995). Therefore, cGMP appears to be an endogenous intracellular cardioprotectant (Pabla *et al.*, 1995).

Cardiac stress adaptation is possibly jeopardized in hyperlipidaemia due to altered NO-cGMP pathway function in vascular and myocardial tissue. Szilvassy and co-workers (2001) found that a cholesterol-enriched diet decreased both vascular NO and cGMP concentrations and increased aortic O_2^- production. Several other studies have also shown that a high cholesterol diet impairs NO-cGMP signalling in both endothelium and non-endothelial cells with a significant decrease in cardiac NO-concentrations (Deliconstantinos *et al.*, 1995; Ferdinandy *et al.*, 1997; Scekerez *et al.*, 1997). Giricz and co-workers (2003) found an increase in O_2^- production with decreased cardiac NO-concentrations in cholesterol-fed rats. However, nitric oxide synthase (NOS) activity was unchanged. These data suggest that NO synthesis was not impaired, but that increased O_2^- production was responsible for the decreased NO-concentrations in the hyperlipidaemic myocardium. Furthermore, hyperlipidaemia stimulates $ONOO^-$ generation in the heart, which leads to myocardial dysfunction (Onody *et al.*, 2003). Newaz and co-workers (2003) showed an antioxidant protection by γ -tocotrienols in hypertensive rats when compared with normotensive control animals. These authors suggested that improved NOS activity in blood vessels and increased NO availability were mediated through the antioxidant properties of γ -tocotrienol where it effectively scavenges the free radicals. Venditti and co-workers (1999) also reported that

vitamin E treatment offers protection against ischaemia/reperfusion-induced oxidative stress, but the precise mechanism of action is unclear.

Diniz and co-workers (2004) showed that changes in dietary fatty acid composition affect cardiac oxidative stress. These authors showed that, despite their beneficial effects on serum lipid concentrations, diets rich in polyunsaturated fatty acids (PUFAs) are deleterious to the heart by increasing cardiac susceptibility to lipid peroxidation. PUFA-fed rats also showed diminished SOD activities as compared to saturated fatty acid (SFA)-fed rats in this particular study. Although many animal feeding studies have shown that fish oil diets rich in n-3 PUFAs prevent ischaemia-induced cardiac arrhythmias (Nair *et al.*, 1997; Kang and Leaf 2000; Jump, 2002), only a few reports have been published on the protective effects of RPO-supplementation against ischaemia/reperfusion injury (Abeywardena *et al.*, 1991; Charnock *et al.*, 1991; Abeywardena and Charnock, 1995).

We speculate that compositional changes in myocardial phospholipid fatty acids during ischaemia may be involved in the regulation of several signal transduction pathways in the heart in direct response to ischaemia/reperfusion-induced injury. One of the best-characterized signal transduction pathways in the heart is the family of mitogen-activated protein kinases (MAPKs). The MAPKs are a family of serine-threonine kinases that are activated in response to a variety of extracellular stimuli (Robinson and Cobb, 1997; Ip and Davis, 1998). Three major MAPKs, including extracellular signal-regulated protein kinase (ERK), p38, and c-Jun N-terminal protein kinase (JNK) have been

implicated in the response to ischaemia and reperfusion in the heart (Bogoyevitch *et al.*, 1996; Knight and Buxton, 1996). All three MAPKs have been shown to play pivotal roles in transmission of signals from cell surface receptors to the nucleus and are involved in cell growth, differentiation and apoptosis (Mansour *et al.*, 1994; Leppa *et al.*, 1998; Nemoto *et al.*, 1998). Another potential target of RPO might be the serine/threonine kinase PKB/Akt. PKB/Akt contains a pleckstrin homology (PH) domain that is part of a slightly larger portion in the NH₂ terminus, called the Akt homology domain. The phosphoinositide 3-kinase (PI3-K) product phosphatidylinositol-3,4-bisphosphate bind *in vitro* directly to the PH domain and increases enzyme activity (Downward, 1998). PKB/Akt has been shown to be activated by factors that stimulate PI3-K including thrombin, platelet-derived growth factor, and insulin (Downward, 1998). There is also increasing evidence that the PKB/Akt pathway participates in ischaemia/reperfusion-induced injury (Brar *et al.*, 2002; Andreucci *et al.*, 2003).

In order to assess the mechanisms of protection, the isolated perfused rat heart model was used to determine whether dietary RPO-supplementation was associated with changes in the regulation of the MAPKs and PKB/Akt in ischaemia/reperfusion. To our knowledge no previous studies have investigated the effect of dietary RPO-supplementation on these signalling pathways during and after ischaemia.

1.1 Aims of the study

The aims of this study were:

- 1) to determine whether dietary RPO-supplementation protects against ischaemia/reperfusion injury in the isolated perfused rat heart from animals on a standard rat chow diet
- 2) to determine whether dietary RPO-supplementation offers the same protection when cholesterol is added to the diet
- 3) to elucidate the mechanisms of protection which include the NO-cGMP signalling pathway, myocardial total phospholipid fatty acid compositional changes during ischaemia and MAPK, PKB/Akt and caspase activities.

CHAPTER 2

LITERATURE REVIEW

2.1 Dietary fats and oils in health

A balanced diet, including oils and fats that supply energy and essential fatty acids is needed for good health. The World Health Organization (WHO) recommends that humans consume about 20-25 kg oils and fats *per capita* per year (Ong and Goh, 2002).

There are many sources of oils and fats, but soybean, palm oil, sunflower seed oil and rape seed oil constitute 60-70% of the world's production (Ong and Goh, 2002). Fats and oils are classified as saturated, monounsaturated and polyunsaturated depending on which fatty acids are dominant. The fatty acid compositions of the most important oils and fats are summarized in Table 2.1.

Plant oils, like coconut oil and palm oil, can be considered highly structured, usually having the sn-2 positional fatty acids unsaturated and the 1,3-fatty acids saturated on the triacylglycerol molecule. The belief that palm oil should be classified nutritionally with saturated fats is therefore debatable. Furthermore, research has also confirmed that palm oil is a non-genetically modified, cholesterol-free, trans-free oil that contains phytonutrients such as α -carotene, β -carotene, vitamin E tocopherols and tocotrienols, lycopene and other carotenoids (Goh *et al.*, 1985; Sundram *et al.*, 2003). These findings merit a re-evaluation of the nutritional properties of palm oil and palm olein

particularly since it has become one of the most important edible oils for human consumption worldwide.

Table 2.1 Saturated, monounsaturated and polyunsaturated fatty acids in palm products, other oils and fats

Oil	SFA	MUFA	PUFA	P/S ratio	U/S ratio	P2/S2 ratio	U2/S2 ratio
Rape seed	5,0	71,0	24,0	4,8	19,0	95,0	166,0
Canola	7,0	61,0	32,0	4,67	13,3	155,0	330,0
Sunflower	11,7	18,0	68,6	5,9	7,4	-	-
Olive	13,0	79,1	7,9	0,6	6,7	10,6	73,0
Corn	13,3	28,4	58,3	4,4	6,5	28,0	39,0
Soybean	16,0	23,5	60,5	3,8	5,3	64,0	85,0
Groundnut	20,0	38,7	41,3	2,1	4,0	14,8	38,0
Cotton seed	27,7	19,8	52,5	1,9	2,6	6,32	8,8
Lard	43,0	47,0	10,0	0,2	1,3	0,04	0,2
Palm olein	46,8	41,5	12,0	0,3	1,1	6,9	22,0
Palm oil	49,5	40,3	9,6	0,2	1,0	1,7	6,7
Red palm oil	50,8	38,3	10,9	0,2	1,0	-	-
Cocoa butter	60,0	36,5	3,4	0,2	0,7	2,3	24,0
Butter	63,4	32,5	4,5	0,1	0,6	0,03	0,2
Hydrogenated soybean^a	64,0+ trans	26,0	4,0	0,1	0,5	-	-
Palm kernel	84,0	14,0	2,0	0,02	0,2	-	-
Coconut	92,2	6,2	1,6	0,02	0,1	0,02	0,05

P/S and U/S: polyunsaturated/saturated and monounsaturated+ polyunsaturated/saturated fatty acid ratios, respectively. P2, S2, U2: polyunsaturated, saturated and total unsaturated fatty acids, respectively, at position sn-2 of the triacylglycerol molecule.

^a Typical sample, saturated 22%, trans fatty acids 42%.

(reproduced from Ong and Goh, 2002)

2.2 Fatty acids

2.2.1 Saturated-, monounsaturated- and polyunsaturated fatty acids in cardiovascular health

Lipids are important dietary constituents and serve in the body as an efficient source of energy when stored in adipose tissue. They are also required by the body for cell structure and membrane function and as a source of precursors

for eicosanoid synthesis. Lipid components like cholesterol and phospholipids regulate membrane-associated functions such as activities of membrane bound enzymes, receptors and ion channels (Clandinin *et al.*, 1991).

Lipids are composed of fatty acids of different chain lengths and degrees of saturation. The differences in chain length and degrees of saturation are known to influence cardiovascular health (Nair *et al.*, 1997). Fatty acids are classified into three types, namely saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). The three major fatty acid families found in mammalian tissue are the n-9 series, the n-6 series and the n-3 series. Figure 2.1 shows the biosynthesis pathway of long chain PUFAs in animals (Pereira *et al.*, 2003).

The main PUFA in the Western diet is linoleic acid, found mostly in vegetable oils such as safflower seed oil, sunflower seed oil, cotton seed oil, corn oil and soybean oil (Nair *et al.*, 1997). The common SFAs in our diet are myristic-, palmitic- and stearic acids derived largely from animal fats, dairy products and manufactured foods. Saturated fatty acids such as palmitic acid (C16:0), can be synthesised from carbohydrates present in the diet (Nair *et al.*, 1997). Palmitic acid can then be elongated to stearic acid (C18:0). The MUFA content of our diet is accounted for by oleic acid (C18:1), the predominant component of canola oil, olive oil and sunola oil (Nair *et al.*, 1997).

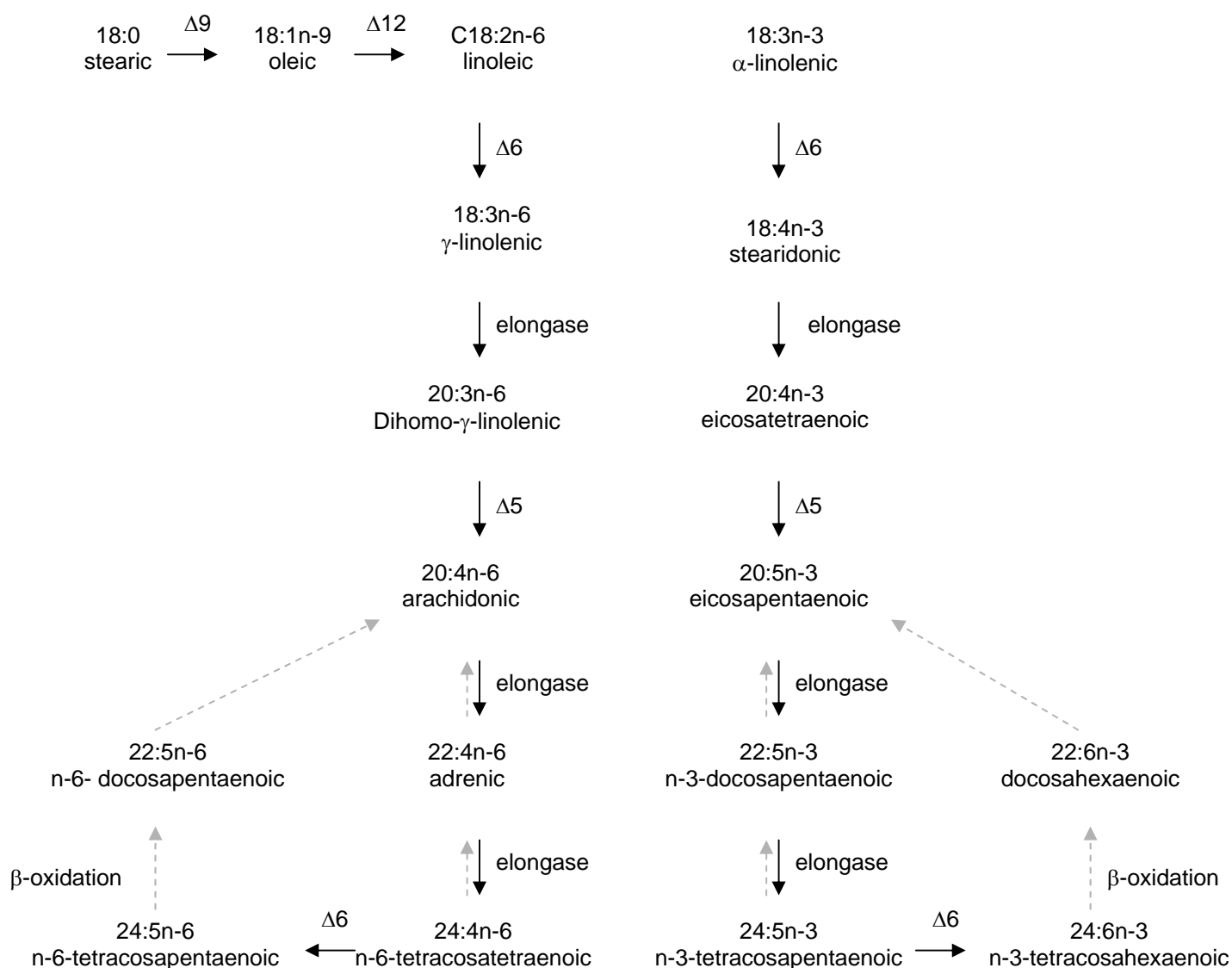


Figure 2.1 Biosynthesis pathway of long-chain PUFAs in animals. The common pathway for synthesis of n-6 and n-3 long chain fatty acids is shown in bold arrows and retroconversion is shown in dashed, gray arrows (reproduced from Pereira *et al.*, 2003)

The Western diet generally includes at least 30-40% of its energy as fat, resulting in a 40% energy intake from lipid sources having SFAs and PUFAs (Schrauwen and Westerterp, 2000). Current recommendations are to increase dietary vegetable oils to increase the ratio of PUFAs to SFAs, lower serum cholesterol and indirectly prevent atherosclerosis (Heyden, 1994). However, one could ask whether the increase in PUFA consumption, despite the decrease in serum cholesterol, is really good for health. Fats high in PUFAs

are more susceptible to oxidation than SFAs. Therefore, PUFAs in the absence of adequate antioxidants increase oxidative stress in the heart and contribute to cardiac dysfunction and myocardial damage by increasing cardiac susceptibility to lipid peroxidation. (Mehta *et al.*, 1994; Esposito *et al.*, 1999; Hart *et al.*, 1999; Droge, 2002; Faine *et al.*, 2002; Novelli *et al.*, 2002; Diniz *et al.*, 2004).

Little is known about the metabolic effects of dietary fatty acids on markers used to evaluate oxidative stress in the heart. However, Diniz and co-workers (2004) showed that rats on a high SFA diet had lower myocardial hydroperoxide concentrations than did PUFA-supplemented animals. This demonstrates the importance of the PUFA:SFA ratio on lipid peroxidation. The readiness with which fatty acids peroxidize is proportional to the number of double bonds, and a positive correlation between the amount of PUFA in the diet and the rate of microsomal lipid peroxidation has been demonstrated in rats (Mehta *et al.*, 1994). The oxidative stability of the cardiac cells is determined by the balance between factors such as PUFAs, which change the PUFA:SFA ratio in the membrane and enhance the sensitivity to lipid peroxidation and the levels of antioxidants (Diniz *et al.*, 2004). These observations support the concept that the sensitivity of cardiac tissue to oxidative stress may depend on dietary factors.

There are many nutritional qualities and benefits of the dietary use of palm oil. Palm oil, like other vegetable oils, is cholesterol free. Having a moderate level of saturation, it does not require hydrogenation for use as a fat component in

foods and, as such, does not contain trans fatty acids (Cottrell, 1991). It is rich in natural antioxidants, which makes it a safe, stable and versatile oil with many positive health and nutritional attributes (Nagendran *et al.*, 2000).

2.2.2 Essential fatty acids

Essential fatty acids (EFAs) are defined as those fatty acids which cannot be biosynthesised or are synthesised in inadequate amounts by animals and humans that require these nutrients for various physiological processes such as growth and maintenance of health (Horrobin, 1990).

Linoleic acid (C18:2n-6; LA) and α -linolenic acid (C18:3n-3; ALA), the parents of the n-6 and n-3 family of fatty acids, respectively, are essential fatty acids that cannot be synthesised in the body and have to be supplied by diet (Figure 2.2; Nair *et al.*, 1997). Linoleic acid is found mostly in vegetable oils such as safflower seed oil, sunflower seed oil, cotton seed oil, corn oil and soybean oil. Other long chain PUFAs, like arachidonic acid (C20:4n-6; AA), are synthesised in human tissue via desaturation and chain elongation from LA which is by far the dominant precursor fatty acid for eicosanoid formation provided by the Western diet. Significant amounts of α -linolenic acid are found in green vegetables and in vegetable oils like linseed oil, canola seed oil and soybean oil. Eicosapentaenoic acid (C20:5n-3; EPA) and docosahexaenoic acid (C22:6n-3; DHA) are synthesised via a series of alternating desaturation and chain elongation steps from ALA and are also found in high concentrations in fish oils. LA and ALA compete for desaturation and chain elongation, therefore

a proper balance is essential to optimize AA and DHA in membranes (Nair *et al.*, 1997).

2.2.3 Physiological role of essential fatty acids

Although many animal feeding studies have shown that fish oil diets rich in n-3 PUFAs prevent ischaemia-induced cardiac arrhythmias (Nair *et al.*, 1997; Kang and Leaf, 2000; Jump, 2002), only a few reports have been published on the protective effects of palm oil-supplementation against ischaemia/reperfusion injury (Abeywardena *et al.*, 1991, Charnock *et al.*, 1991; Abeywardena and Charnock, 1995). Several mechanisms for protection by fish oil diets have been proposed. However, all the mechanisms suggested appear to be interrelated and it is not known whether their effects are independent or are compounded and also in what sequence they take place (Nair *et al.*, 1997).

The polyunsaturated fatty acids, arachidonic acid (C20:4n-6; AA) and eicosapentaenoic acid (C20:5n-3; EPA) are metabolised through cyclooxygenase and lipoxygenase pathways to form eicosanoids, including prostaglandins (PGs), thromboxanes (TXs), prostacyclins (PGIs) and leukotrienes (LTs) (Figure 2.2).

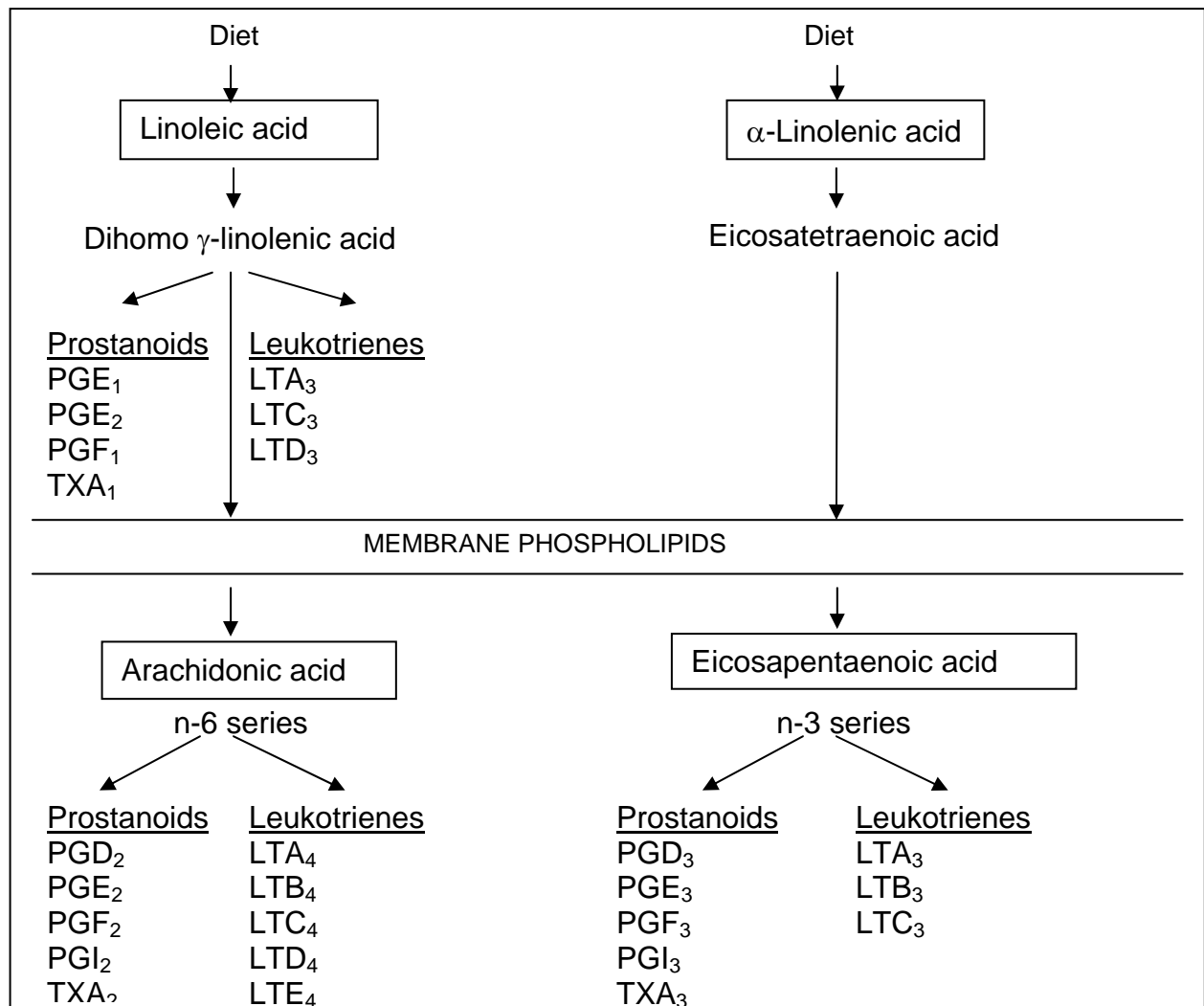


Figure 2.2 Biosynthesis of eicosanoids from dietary fatty acids. LT=leukotrienes; PG=prostaglandins; TX=thromboxane (reproduced from Nair *et al.*, 1997)

Eicosanoids are known to have a variety of cardiovascular effects: 1) all prostaglandins derived from AA are arrhythmogenic, of which PGF₂ is the most potent, 2) PGE₁ from Dihomo-γ-linolenic acid (C20:3n-6; DGLA) appears to have concentration-dependent effects with low concentrations being antiarrhythmic and high concentrations arrhythmogenic, 3) the precursor fatty

acids (free n-6 series AA and EPA n-3 series) are able to prevent eicosanoid-induced arrhythmias, 4) the eicosanoids derived from EPA are generally less arrhythmogenic, 5) lipoxygenase metabolites of both AA and EPA are neither arrhythmogenic nor antiarrhythmic (Li *et al.*, 1997; Yunyuan *et al.*, 1997). The role of AA in arrhythmias is of particular interest. Most investigations on the link between fish oils and cardiovascular disease have demonstrated competition between AA and EPA to become substrates in the production of eicosanoids. When fish oils are included in the diet, the n-3 PUFAs (EPA and DHA) compete with AA in several ways: 1) they inhibit delta 6 ($\Delta 6$) activity to decrease AA biosynthesis (Garg *et al.*, 1988), 2) they compete with AA for the sn-2 position in triacylglycerols and membrane phospholipids and thereby reduce plasma and cellular levels of AA (Siess *et al.*, 1988), 3) EPA competes with AA as the substrate for the cyclooxygenase enzyme thus inhibiting the production of thromboxane A₂ (TXA₂) by platelets (Fischer and Weber, 1984).

Research has shown that dietary supplementation of different edible oils may influence cardiovascular function due to compositional changes in the PUFAs of the myocardial membrane phospholipids after ischaemia and reperfusion (Abeywardena *et al.*, 1991; Abeywardena and Charnock, 1995). The presence of fish oil in the diet results in increased incorporation of n-3 PUFAs (EPA and DHA), mainly at the expense of n-6 unsaturated arachidonic acid. The significant increase in DHA associated with fish oil supplementation is likely to be due not only to a direct incorporation of DHA from the diet, but also to an increased elongation and further desaturation of EPA (Abeywardena and Charnock, 1995). Both fish oil- and RPO-supplementation caused a significant

inhibition of myocardial thromboxane A₂ production. Abeywardena and co-workers (1991) speculated that n-3 PUFAs (EPA and DHA) might act as specific inhibitors of thromboxane synthases, whereas the effect of RPO is unlikely to be mediated via fatty acids. Charnock and co-workers (1991) investigated the effect of long-term feeding with various dietary fats and oils on cardiac arrhythmias in an animal model. These authors showed that dietary supplementation with saturated animal fat (SF) increased the susceptibility to develop cardiac arrhythmias under ischaemic stress whereas the polyunsaturated fatty acids of sunflower seed oil (SSO) reduced this susceptibility. RPO-supplementation produced results that lay between those for the SF and SSO groups. Furthermore, the number of animals displaying severe ventricular fibrillation was reduced after RPO-supplementation when compared with SF feeding (Charnock *et al.*, 1991). From the limited data available, it is unclear whether these results are related to the ratio of polyunsaturated to saturated fatty acids of the diets, or to the fatty acid composition of the myocardial membranes. These effects may even be mediated by differential actions of the dietary fats on myocardial eicosanoid production. In addition, it has been suggested by Gapor and co-workers (1989) that palm oil antioxidants in a palm oil/fish oil-supplementation may also prove to be useful for protection of the less stable polyunsaturated fatty acids in fish oils.

2.3 Cholesterol-enriched diets

2.3.1 Introduction to cholesterol-enriched diets

A high-cholesterol diet is regarded as an important factor in the development of cardiovascular disease, since it leads to development of hyperlipidaemia, atherosclerosis and ischaemic heart disease (Puskas *et al.*, 2004).

2.3.2 Mechanisms of myocardial effects of hyperlipidaemia

The exact biochemical mechanisms of the direct effects of high-cholesterol diet hyperlipidaemia on the myocardium are still a question of debate. However, the following mechanisms have been shown to play a role in the cardiac effects of hyperlipidaemia: 1) inhibition of the mevalonate pathway (Ferdinandy *et al.*, 1998), 2) decrease in NO bioavailability and cGMP metabolism (Ferdinandy *et al.*, 1997; Szekeres *et al.*, 1997), 3) increase in free radical and peroxynitrite production (Onody *et al.*, 2003), 4) inhibition of heat shock response (Csont *et al.*, 2002), 5) expression of oxidized low-density lipoprotein receptors which induced apoptosis (Chen *et al.*, 2002). Recent studies identified gene activity changes in atherosclerotic plaques in human and animal blood vessels and rat hearts (Puskas *et al.*, 2004).

2.4 Palm oil

2.4.1 Introduction to palm oil

Crude palm oil is produced from the fruit of the *Elaeis guineensis* tree (Nagendran *et al.*, 2000; Sundram *et al.*, 2003) and has a long history of food use dating back over 5 000 years. Palm oil is one of the 16 edible oils

possessing an FAO/WHO Food standard under the Codex Alimentarius Commission Programme, which comprises 122 member countries (Codex Alimentarius, 1983). Palm oil is a traditional food source native to West Africa. From its origin in Africa, oil palm has crossed the oceans of the world to become an important plantation crop in countries like Malaysia. Here it emerged as the most prolific oil bearing crop in the world. A single tree has an economic lifespan of 20-30 years and annually bears 10-12 fruit bunches, each weighing between 20-30 kg.

2.4.2 Composition of palm oil

Crude palm oil consists of glycerides and small quantities of non-glyceride components including free fatty acids, trace metals, moisture and impurities, and minor components. The minor components in crude palm oil are carotenoids, tocopherols, tocotrienols, sterols, phospholipids, squalene and hydrocarbons (Goh *et al.*, 1985, Sundram *et al.*, 2003). Of these the carotenoids, tocopherols and tocotrienols are the most important minor components and together they contribute to the stability and nutritional properties of palm oil (Ooi *et al.*, 1996). A novel process involving pre-treatment of crude palm oil, followed by deacidification and deodorization using molecular distillation, can be used to produce a carotene-rich refined edible palm oil. The product is a refined red palm oil that meets standard refined edible oil specifications and retains up to 80% of the carotene and vitamin E originally present in the crude palm oil. The oil contains no less than 500 ppm carotene, 90% of which is present as α - and β -carotene. The vitamin E content is about 500 ppm of which 70% is in the form of tocotrienols (mainly as α -, β -

and γ tocotrienols). Other important minor components present in this oil are ubiquinones and phytosterols (Nagendran *et al.*, 2000; Sundram *et al.*, 2003).

2.4.3 Modulation of lipids and lipoproteins by dietary palm oil-supplementation

Since a high blood cholesterol level is a risk factor for cardiovascular disease, numerous studies have investigated the effects of dietary lipids on cholesterol levels. Research has shown that most unsaturated fatty acids have a cholesterol-lowering effect, whereas saturated fatty acids increase serum cholesterol (Diniz *et al.*, 2004).

Several clinical trials evaluated the effect of palm oil on blood lipids and lipoproteins and showed that palm oil does not raise serum total cholesterol (TC) or LDL cholesterol concentrations to the extent expected based on its fatty acid composition (Chandrasekharan, 1999; Theriault *et al.*, 1999; Kritchevsky *et al.*, 2000; Sundram and Basiron). These studies suggest that the cholesterolaemic effects of palm oil depend on several factors including fatty acid composition (Table 2.2). The saturated fatty acids of palm oil consist of palmitic acid (44%) and stearic acid (5%), and the unsaturated fatty acids are oleic acid (39%) and linoleic acid (10%) (Ong and Goh, 2002).

Many studies have confirmed the nutritional value of palm oil as a result of the high monounsaturation at the crucial sn-2 position of the oil's triacylglycerols, making this oil as healthy as olive oil. The monounsaturated and polyunsaturated fatty acids constitute 87% of the total fatty acids at the sn-2

position (Ong and Goh, 2002). Comparison of palm oil with a variety of monounsaturated edible oils including rape seed, canola and olive oils has shown that serum cholesterol and LDL-cholesterol are not elevated by palm oil (Sundram and Basiron). Furthermore, substitution of palmitic acid from palm oil for the lauric acid and myristic acid combination of palm kernel oil and coconut oil leads to a decrease in serum cholesterol and LDL-cholesterol (Ong and Goh, 2002).

Research has shown that the contribution of dietary fats to blood lipids and cholesterol modulation is a sequence of the digestion, absorption and metabolism of the fats. Lipolytic hydrolysis of palm oil's glyceride containing predominantly oleic acid at the sn-2 position and palmitic and stearic acids at the sn-1 and sn-3 positions allow for the ready absorption of the sn-2 monoglycerols, while the saturated free fatty acids remain poorly absorbed. Therefore, dietary palm oil in balanced diets (when a moderate-fat, moderate-cholesterol diet is consumed) generally reduces blood cholesterol and triacylglycerol, while raising the HDL-cholesterol (Ong and Goh, 2002).

Apart from these fatty acids (Table 2.2), there is evidence that the tocotrienols in palm oil products may have a hypocholesterolaemic effect. This is mediated by the ability of the tocotrienols to suppress 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis (Khor *et al.*, 1995; Theriault *et al.*, 1999; Sundram and Basiron) (Figure 2.3).

Table 2.2 Fatty acid composition of palm oil and its effects on blood cholesterol

Fatty acid	Composition (%)	Effects on blood cholesterol
Lauric acid 12:0	0,2	Negative or neutral
Myristic acid 14:0	1,1	Negative
Palmitic acid 16:0	44,3	Neutral or slightly negative
Stearic acid 18:0	4,6	Neutral
Oleic acid 18:1	39,0	Positive
Linoleic acid 18:2	10,5	Positive
Others 16:1;18:3	0,3	Positive
Total in palm oil	100,0	Positive

Palm oil and palm olein contain insignificant amounts of cholesterol-elevating saturated fatty acids (12:0 and 14:0); negative means cholesterol-raising; positive means no effect or decreasing cholesterol (reproduced from Ong and Goh, 2002)

2.4.4 Epidemiological studies: lipids

After almost 50 years of little concern about the increased consumption of hydrogenated fats at the expense of saturated fatty acids, Mensink and Katan (1990) showed that *trans* fatty acids increased serum total and LDL-cholesterol and decreased the beneficial HDL-cholesterol. Research confirmed these results which have since been seen as the standard of comparison that leads to the conclusion that *trans* fatty acids increased the risk for cardiovascular disease similarly to saturated fatty acids (Wood *et al.*, 1993; Sundram *et al.*, 1997). The Harvard research group led by Willet and co-workers (1999) spearheaded studies elucidating the effects of *trans* fatty acids using

epidemiological data from the Nurses Health Study consisting of 85 095 women.

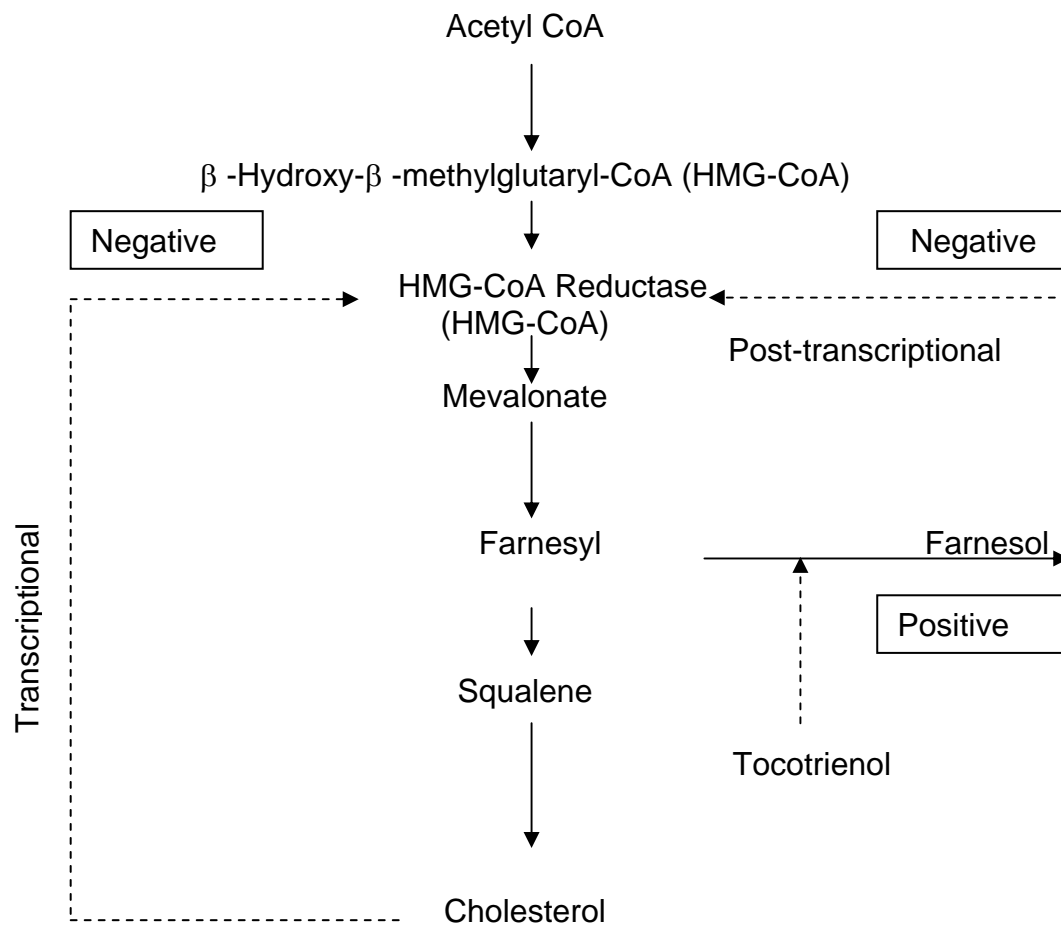


Figure 2.3 Mechanism of the cholesterol suppressive action of tocotrienol. Tocotrienols prenylated side-chain is thought to induce prenyl pyrophosphate pyrophosphatase that catalyzes the dephosphorylation of farnesyl with a concomitant increase in cellular farnesol. Farnesol, in turn, downregulates HMG CoA reductase activity by a post-transcriptional process involving protein degradation. This action is different from that of cholesterol, which exerts a feedback transcriptional effect on HMG-CoA reductase activity (reproduced from Theriault *et al.*, 1999).

They examined the association between *trans* fatty acids and incidence of non-fatal myocardial infarction or death from coronary heart disease in these women who were followed for eight years. A positive and significant association between *trans* fatty acids and Coronary Heart Disease (CHD) was apparent. A follow-up study in 239 patients (Ascherio *et al.*, 1994) also showed

a positive association between *trans* fatty acids (in margarine) and myocardial infarction. *Trans* fatty acid intake was associated with increased serum total and LDL-cholesterol and negatively related to HDL-cholesterol in men suffering a myocardial infarction.

The relative risk for Cardiovascular Disease (CVD) was increased by 27% as a result of *trans* fatty acid consumption. These studies clearly established an association between *trans* fatty acid consumption and increased incidence and death from CVD and it was estimated that almost 80 000 deaths in the United States alone are associated with continued consumption of foods rich in *trans* fatty acids.

2.4.5 Cardiovascular protection offered by palm oil components

2.4.5.1 Carotenoids

A comparison between Carotino palm oil and other vegetable oils (Table 2.3) shows that palm oil is the only one that is naturally rich in carotenoids. It is 15 times richer in carotenes than carrots and contains 50 times more carotenes than tomatoes (Kamen, 2000).

Table 2.3 Comparison between Carotino palm oil and other vegetable oils

	Carotino Premium (Palm oil)	Carotino classic (Palm oil)	Sunflower seed oil	Safflower seed oil	Corn oil	Olive oil
Vitamin E mg/kg	80	50	39	17,4	20,7	7,6
Carotene mg/kg	50	12,5	0	0	0	0

(reproduced from Kamen, 2000)

Carotenoids are a group of red, orange and yellow pigments found in plant foods, particularly fruit and vegetables. β -Carotene is an effective antioxidant

as it is one of the most powerful singlet oxygen quenchers. It can dissipate the energy of singlet oxygen, thus preventing this active molecule from generating free radicals (Bagchi and Puri, 1998).

2.4.5.2 Vitamin E (tocopherols and tocotrienols)

Vitamin E is the collective name for eight compounds, namely four tocopherols and four tocotrienols (Bagchi and Puri, 1998). The biological activity of vitamin E has generally been associated with its well-defined antioxidant property, especially against lipid peroxidation in biological membranes (Theriault *et al.*, 1999). Vitamin E is highly lipophilic and is believed to be the major lipid-soluble chain-breaking antioxidant found in blood plasma and protects polyunsaturated fatty acids in cell membranes from peroxidation (Bagchi and Puri, 1998). Within biological membranes, vitamin E is believed to intercalate with phospholipids and provide protection to PUFAs. Oxidation of PUFAs leads to disturbances in membrane structure and function and is damaging to cell structure. Vitamin E is highly efficient at preventing the auto-oxidation of lipids and it appears as if this is its primary function in biological tissue (Burton and Ingold, 1981). It is a singlet oxygen quencher and neutralises these highly reactive and unstable molecules (Kamal-Eldin and Appelqvist, 1996). α -Tocopherol is considered to be the most active form of vitamin E. However, research has suggested tocotrienol to be a better antioxidant (Serbinova *et al.*, 1991; Suzuki *et al.*, 1993).

More recently, alternative non-antioxidant functions of vitamin E have been proposed and in particular that of a “gene regulator”. Effects of vitamin E have

been observed at the level of mRNA or protein and could be consequent to regulation of gene transcription, mRNA stability, protein translation, protein stability and post-translational events (Ricciarelli *et al.*, 2001; Assi *et al.*, 2002). Moreover, tocotrienol has been shown to possess novel hypocholesterolaemic effects together with an ability to reduce the atherogenic apolipoprotein B and lipoprotein(a) serum concentrations (Hood, 1995). In addition, tocotrienol has been suggested to have an anti-thrombotic and anti-tumour effect indicating that tocotrienol may serve as an effective agent in the prevention and/or treatment of cardiovascular disease and cancer (Guthrie *et al.*, 1995; Qureshi *et al.*, 1997).

Vitamin E is known to afford protection against ischaemia and reperfusion injury (Bagchi and Puri, 1998). Epidemiological evidence strongly associates high vitamin E intake with reduced risk of coronary heart disease. Stephens and co-workers (1996) showed that vitamin E treatment significantly reduced the risk of cardiovascular deaths as well as non-fatal myocardial infarctions.

While all vegetable oils have tocopherols, palm oil has also tocotrienols in abundance. In fact, among vegetables, palm oil is the only rich source of tocotrienols (Kamen, 2000). In rat ischaemia/reperfusion studies, α -tocotrienol protected more efficiently against oxidative stress than α -tocopherol as shown by improved reperfusion function recovery in a Langendorff perfused rat heart (Serbinova *et al.*, 1992). This higher antioxidant activity of the tocotrienols has been attributed to a number of mechanisms including efficient interaction with free radical species, higher recycling efficiency of the chromanoxyl radical and

uniform distribution of tocotrienols in membrane bilayers (Serbinova *et al.*, 1991; Theriault *et al.*, 1999).

Yoshida and co-workers (2003) carried out a comparative study on the action of tocopherols and tocotrienols as antioxidants and found that: 1) the tocopherols and tocotrienols exerted the same effects on free radical scavenging and lipid-peroxidation in solution and liposomal membranes, 2) tocopherols increased the rigidity of liposomal membranes more significantly than tocotrienols, 3) tocopherols and tocotrienols showed similar mobilities within the liposomal membranes, but tocotrienols were more readily transferred between the membranes and incorporated into the membranes than tocopherols. These findings are in agreement with data reported previously by Sen and co-workers (2000) that tocotrienols were more readily incorporated into cultured cells than tocopherols. Therefore, tocotrienols appear to be more effective antioxidants than tocopherols due to higher uptake.

Although both tocopherols and tocotrienols are natural antioxidants, the apparent 'antioxidant activities' of tocopherols and tocotrienols may vary depending on the experimental conditions applied. The inconsistent results reported previously for the antioxidant activities of tocopherols and tocotrienols may be ascribed partly to the different experimental conditions and evaluation methods used (Yoshida *et al.*, 2003).

Toxicological and pharmacological studies in rats found that palm tocotrienols are safe without adverse side effects when consumed at doses as high as

2500 mg per kg body weight. The recommended human dosage is 50-100 mg per 60 kg body weight (Oo *et al.*, 1992).

2.4.5.3 Ubiquinones

Crude palm oil contains small quantities of ubiquinones of which coenzyme Q₁₀ (CoQ₁₀) is the most common. Although it is present at a relatively low concentration in crude palm oil, CoQ₁₀ has been reported to boost the immune system, relieve angina and afford protection against heart disease and reduction of high blood pressure (Nagendran *et al.*, 2000).

CoQ₁₀ plays a major role in the mitochondrial electron-transport system and as an antioxidant protects the ischaemic/reperfused myocardium in rats (Hano *et al.*, 1994). Yokoyama and co-workers (1996) proposed that CoQ₁₀ is a free radical scavenger and preserves coronary vessel mechanical function following ischaemia/reperfusion injury via a direct antioxidant mechanism.

2.4.6 Palm oil protection against breast cancer

Components of palm oil such as coenzyme Q₁₀, tocotrienols and vitamin E succinate have possible protective effects against breast cancer (Guthrie *et al.*, 1995).

2.4.6.1 Coenzyme Q₁₀ (CoQ₁₀) protection

Since the 1960's studies have shown that cancer patients often have decreased blood levels of CoQ₁₀ due to increased consumption of this coenzyme by oxygen free radical scavenging. In particular, breast cancer patients who underwent radical mastectomy were found to have significantly decreased tumour concentrations of CoQ₁₀ compared to levels in normal surrounding tissues. Therefore, CoQ₁₀ may have a protective effect on breast tissue (Portakal *et al.*, 2000).

CoQ₁₀ has also demonstrated promise in treating breast cancer. In a clinical study 32 patients were treated with CoQ₁₀ (90 mg) in addition to other antioxidants and fatty acids (Lockwood *et al.*, 1994). Six of these patients showed partial tumour regression. In one of these cases the dose of CoQ₁₀ was increased to 390 mg and within one month the tumour was no longer palpable. Within two months the mammography confirmed the absence of tumour. In another case, a patient took 300 mg of CoQ₁₀ for residual tumour tissue (post non-radical surgery) and within three months there was non-residual tumour tissue. This overt complete regression of breast tumours in these two cases, coupled with further reports of disappearance of breast cancer metastases (liver and elsewhere) in several other cases (Lockwood *et al.*, 1995), demonstrates the potential of CoQ₁₀ therapy in breast cancer.

Furthermore, there are promising results for the use of CoQ₁₀ in protecting against heart damage related to chemotherapy. Animal studies found that

CoQ₁₀ could reduce the adverse cardiac effects of these chemotherapy drugs (Folkers, 1996).

2.4.6.2 Tocotrienol protection

Tocotrienols elicit powerful anticancer properties and studies have confirmed tocotrienol activity to be much stronger than that of tocopherols (Schwenke *et al.*, 2002). Tocotrienols possess the ability to stimulate the selective killing of cancer cells through programmed cell death (apoptosis) and to reduce cancer cell proliferation while leaving normal cells unaffected (Kline *et al.*, 2001).

2.4.6.3 Vitamin E succinate protection

Vitamin E succinate, a derivative of fat-soluble vitamin E, has been shown to inhibit tumour cell growth *in vitro* and *in vivo* (Cameron *et al.*, 2003). Since vitamin E is considered the main chain breaking lipophilic antioxidant in serum and tissue, its role as a potential chemopreventative agent and its use in the adjuvant treatment of aggressive human breast cancer appears reasonable.

2.4.7 Effects of palm oil-supplementation on NO-cGMP signalling

Nitric Oxide (NO) is an important regulator of both cardiac and vascular function and tissue reperfusion (reviewed by Ferdinandy and Schultz, 2003). Little is known about the effects and possible protective mechanisms of palm oil-supplementation against myocardial ischaemia/reperfusion injury, particularly pertaining to NO-cGMP signalling.

2.5 Role of nitric oxide in myocardial ischaemia and reperfusion

2.5.1 Introduction to nitric oxide cardiovascular protection

Ischaemic heart disease, which is characterized by insufficient blood supply to regions of the myocardium, develops as a consequence of many pathological conditions including hypertension, atherosclerosis, hyperlipidaemia and diabetes. The development of cardioprotective agents which improve myocardial function, decrease the incidence of arrhythmias, lessen the necrotic tissue mass and delay the onset of necrosis during ischaemia/reperfusion, is of great clinical importance (reviewed by Ferdinandy and Schultz, 2003).

Over the past decade many studies have focused on the role of NO in myocardial ischaemia. The overwhelming majority of the studies published, support a cytoprotective role for NO (either endogenous or exogenous) in myocardial ischaemia/reperfusion injury, both *in vitro* and *in vivo* (reviewed by Bolli, 2001).

Sources of basal NO production by Ca^{2+} -dependent NO synthases (NOS) include coronary endothelium, endocardial endothelium, cardiac nerves and cardiomyocytes of the normal heart (Curtis and Pabla, 1997). NO serves a number of important physiological roles in the regulation of cardiac function including coronary vasodilation, inhibiting platelet and neutrophil actions, antioxidant effects, modulation of cardiac contractile function and inhibition of cardiac contractile energy consumption (Hare and Comerford, 1995; Xie and Wolin, 1996).

NO offers cardioprotection against ischaemia/reperfusion injury (Maulik *et al.*, 1995; Williams *et al.*, 1995; Araki *et al.*, 2000; Bolli, 2001). Several mechanisms of cardioprotection include stimulation of soluble guanylate cyclase and thus bringing about reduction of Ca^{2+} , partly through activation of cGMP-dependent protein kinase and termination of chain propagating lipid radical reactions caused by oxidative stress (Rubbo *et al.*, 1994).

However, NO is detrimental when it is combined with superoxide (O_2^-) to form peroxynitrite (ONOO^-), which rapidly decomposes to highly reactive oxidant species leading to tissue injury (Figure 2.4). There is a critical balance between cellular concentrations of NO, O_2^- and superoxide dismutase (SOD) which physiologically favours NO production, but in pathological conditions such as ischaemia and reperfusion, results in ONOO^- formation (reviewed by Ferdinandy and Schultz, 2003). Illarion and co-workers (2002) reviewed the role of reactive oxygen species, focusing mainly on superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot\text{OH}$), which have long been implicated in the pathogenesis of ischaemia/reperfusion injury. These oxygen free radicals can react with nucleic acids, proteins and lipids, resulting in damage to the cell membrane or intracellular organelles. Vitamin E acts as a free radical scavenger that can react with oxygen, superoxide anion radicals and hydroxyl radicals (Wall, 2000; Abuda *et al.*, 2004) (Figure 2.4).

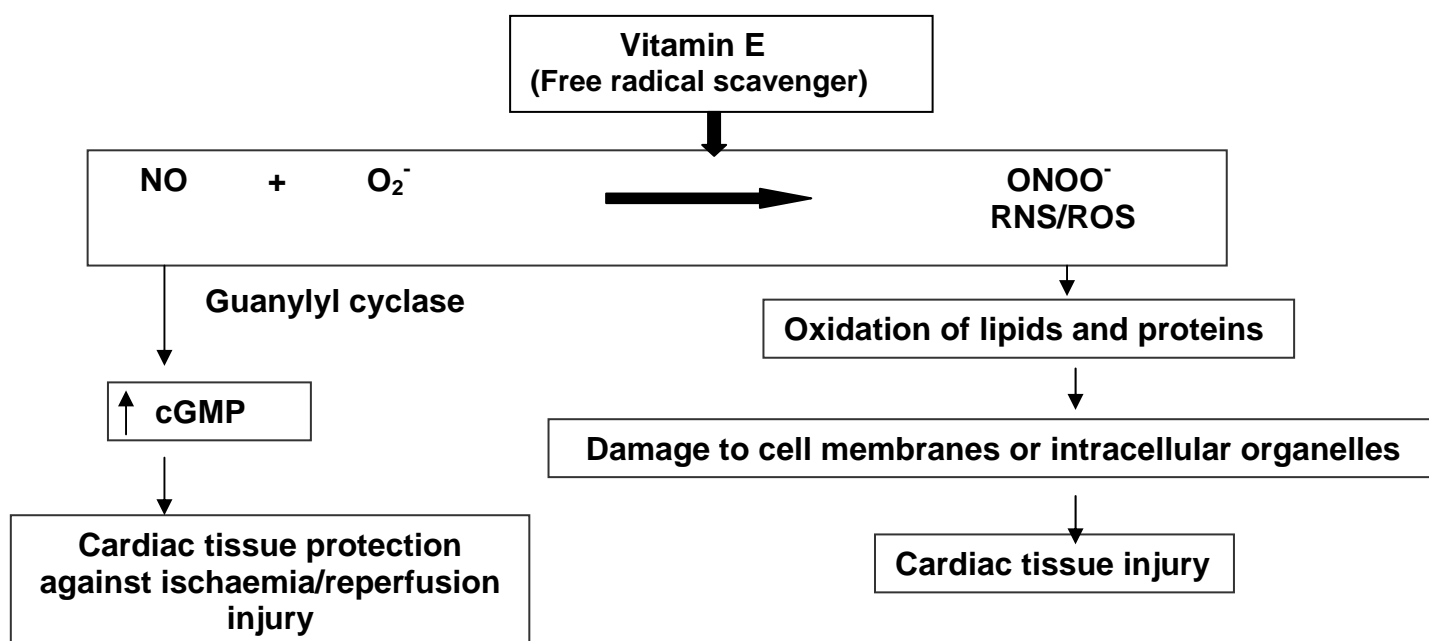


Figure 2.4 Cellular mechanisms of nitric oxide (NO), superoxide (O_2^-), and peroxynitrite ($ONOO^-$) actions.

2.5.2 Mechanisms of cardiovascular protective effect of nitric oxide

The precise mechanism(s) whereby NO protects the myocardium against ischaemia/reperfusion injury remains unclear. Some of the many hypotheses that have been put forward will be discussed.

2.5.2.1 NO-cGMP signalling

NO and/or its second messenger, cGMP, have been shown to exert a number of actions that are expected to be beneficial during myocardial ischaemia including inhibition of Ca^{2+} influx into myocytes, antagonism of the effects of β -adrenergic stimulation, decreasing myocardial contractility and opening of sarcolemmal K_{ATP} channels. The reduced Ca^{2+} current may alleviate the Ca^{2+}

overload associated with acute myocardial ischaemia - one of the major mechanisms of ischaemic injury (reviewed by Bolli, 2001).

NO is known to increase myocardial cGMP and it can be speculated that the protective effect of NO is related to a mechanism secondary to the stimulation of guanylyl cyclase within the vascular wall or in ventricular myocytes (Beresewics *et al.*, 1995; Maulik *et al.*, 1995; Depré *et al.*, 1996) (Figure 2.4). NO donors administered during ischaemia possibly protect the myocardium by increasing tissue cGMP and decreasing cytosolic Ca^{2+} overload (Du Toit *et al.*, 2001). Du Toit and co-workers (2001) found that nitric oxide donor treatment reduces ischaemia/reperfusion injury by increasing cGMP concentrations and suggested that the cAMP-to-cGMP ratio might play an important role in cardioprotection. Maulik and co-workers (1995) showed that NO plays a significant role in transmembrane signalling in the ischaemic myocardium. This group suggested that NO signalling is switched off due to inactivation of NO by reactive oxygen species and was the first to suggest that reactive oxygen species may alter NO-cGMP signalling.

Increases in cAMP concentrations associated with ischaemia would increase Ca^{2+} concentrations and exacerbate ischaemic/reperfusion injury (Du Toit *et al.*, 2001). In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP induced increase in slow inward calcium current, thus leading to a decrease in cytosolic calcium levels (Sumii and Sperelakis, 1995). Therefore, cGMP appears to be an endogenous intracellular cardioprotectant (Pabla *et al.*, 1995; Pabla and Curtis, 1995).

2.5.2.2 Cholesterol-enriched diet and NO-cGMP signalling

It is well known that high cholesterol concentrations influence the NO-cGMP signalling pathway. Therefore, cardiac stress adaptation is possibly jeopardized in hyperlipidaemia due to altered NO-cGMP pathway function in vascular and myocardial tissue. Szilvassy and co-workers (2001) found that a cholesterol-enriched diet decreased both vascular NO and cGMP concentrations and increased aortic O_2^- and $ONOO^-$ production. Several other studies have also shown that a high-cholesterol diet impairs NO-cGMP signalling in both endothelium (Deliconstantinos *et al.*, 1995) and non-endothelial cells with a significant decrease in cardiac NO concentrations (Ferdinandy *et al.*, 1997; Szekeres *et al.*, 1997). Giricz and co-workers (2003) found that although cardiac NO-concentration in cholesterol-fed rats was decreased, nitric oxide synthase (NOS) activity was unchanged which may suggest that NO synthesis was not impaired. The mechanism leading to decreased cardiac NO level in hyperlipidaemia remains unknown. However, it is well known that hyperlipidaemia leads to increased production of reactive oxygen species (ROS) in the vasculature (Kojda and Harrison, 1999). For example, hyperlipidaemia stimulates $ONOO^-$ generation in the heart, which leads to myocardial dysfunction (Onody *et al.*, 2003). It could be speculated that increased O_2^- production (Giricz and co-workers, 2003) is responsible for the decreased NO concentrations in the hyperlipidaemic myocardium since NADPH oxidase activity due to hyperlipidaemia is a major source of increased O_2^- production (Warnholtz *et al.*, 1999).

Natural antioxidants can act as scavengers of damaging oxygen free radicals (Cotrell, 1991; Chandrasekharan, 1999; Theriault *et al.*, 1999; Kritchevsky, 2000; Wall, 2000). Vitamin E acts as a free radical scavenger that can react with oxygen, superoxide anion radicals and hydroxyl radicals (Wall, 2000; Abuda *et al.*, 2004). Due to its lipid solubility, it is predominantly a chain breaking antioxidant within the lipoprotein (Wall, 2000; Abuda *et al.*, 2004). Chow and co-workers (2002) reported that dietary vitamin E is capable of reducing the production and/or availability of not only O_2^- , but also NO and ONOO⁻. By reducing available O_2^- and NO, vitamin E may alleviate nitric toxicity via reduced formation of reactive ONOO⁻. However, it is not clear if the action of vitamin E to reduce the generation of O_2^- and other ROS species is independent of its antioxidant function (Chow and Hong, 2002). Newaz and co-workers (2003) showed an antioxidant protection by γ -tocotrienols in hypertensive rats when compared with control animals. These authors suggested that improved NOS activity in blood vessels and increased NO availability were mediated through the antioxidant properties of γ -tocotrienol where it effectively scavenges the free radicals. Venditti and co-workers (1999) also reported that vitamin E treatment offers protection against ischaemia/reperfusion-induced oxidative stress. However, the precise mechanism of action is unknown.

2.5.2.3 Antioxidant properties of nitric oxide

By virtue of its antioxidant properties, NO offers protection specifically through its ability to attenuate the deleterious free radical actions of O_2^- and to terminate $ONOO^-$ mediated lipid radical chain propagation (Rubbo *et al.*, 1994).

2.5.2.4 Nitric oxide and production of cytoprotective prostanoids

A previously unrecognized mechanism by which NO protects the ischaemic myocardium has recently emerged, namely, stimulation of cyclooxygenase-2 (COX-2) activity with consequent production of cytoprotective prostanoids such as prostaglandin (PGE_2 and PGI_2) (Shinmuri *et al.*, 2000).

2.5.2.5 Therapeutical potential of nitric oxide

Research has shown that NO plays a fundamental biological role in protecting the heart against ischaemia/reperfusion injury. The recognition that production of NO represents nature's own protective mechanism against ischaemia offers fertile practical implications. Many opportunities loom on the horizon for enhancing NO availability in a manner that would be therapeutically desirable (Bolli, 2001).

2.6 Mitogen-activated protein kinases (MAPKs)

2.6.1 Apoptosis

Programmed cell death (apoptosis) is essential to many biological processes in multicellular organisms including embryonic development, immune responses, tissue homeostasis and normal cell turnover (reviewed by Lin, 2003).

Research has shown that both necrosis and apoptosis play a significant role in myocardial ischaemia/reperfusion injury (Kajstura *et al.*, 1996). Stress conditions such as ischaemia (especially when followed by reperfusion) and oxygen free radicals can elicit cardiomyocyte apoptosis, as has been demonstrated in both cell cultures and isolated perfusion (reviewed by Feuerstein and Young, 1999).

2.6.2 Signalling pathways and apoptosis

Regulatory mechanisms controlling proliferation, differentiation or apoptosis of cells involve intracellular protein kinases that can transduce signals detected on the cell's surface into changes in gene expression. There is growing evidence that multiple mitogen-activated protein (MAP) kinases and protein kinase B (PKB/Akt) are activated during ischaemia and/or reperfusion and may contribute to the structural and functional changes after myocardial ischaemia/reperfusion injury (Abe *et al.*, 2000). However, reports on their precise roles are still conflicting (reviewed by Steenbergen, 2002).

Three major MAP kinase cascades that have been extensively studied in the heart include extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNK 1 and JNK 2), and p38 kinases, of which the p38 α and p38 β isoforms are found to play a role in myocardial tissue (Takeishi *et al.*, 1999).

MAP kinases are proline-directed serine/threonine kinases that are activated in response to a wide variety of stimuli including growth factors, G-protein coupled receptors and environmental stresses (Robinson and Cobb, 1997; Sugden and Clerk, 1998). The MAP kinases themselves require dual phosphorylation on a Threonine-X-Tyrosine (Thr-X-Tyr) motif to become active

and each of these enzymes is a target for discrete but closely related phosphorylation cascades in which the sequential activation of three kinases constitutes a common signalling mode (Gartner *et al.*, 1992; Fanger *et al.*, 1997; Siow *et al.*, 1997).

2.6.3 Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

modulate mitogen-activated protein kinases (MAPK) activity

Mirnikjoo and co-workers (2001) reported that n-3 fatty acids modulate activation of extracellular signalling pathways by inhibiting the *in vitro* activities of cAMP-dependent protein kinase, protein kinase C (PKC), Ca²⁺/calmodulin dependent protein kinase 2 and the mitogen activated protein kinases (MAPKs). These authors showed that DHA and EPA act as broad-spectrum kinase inhibitors, blocking not only the activity of PKC but also the activity of MAPK. These results were confirmed by Denys and co-workers (2002) who showed that EPA and DHA regulate human T-cell function by modulating MAPK enzyme activity. Other studies have shown that EPA also inhibits hypoxia-reoxygenation-induced injury by attenuating upregulation of MMP-1, thereby reducing phosphorylation of p38 (Cheng *et al.*, 2003).

2.6.4 The major multiple mitogen-activated protein kinase (MAPK) and PKB/Akt signalling pathways

2.6.4.1 p38 MAPK

At least 6 isoforms of the p38 MAPK group have been cloned, purified and characterized, namely p38 α 1/ α 2 (Han *et al.*, 1994; Lee *et al.*, 1994), p38

$\beta 1/\beta 2$ (Jiang *et al.*, 1996), p38 γ (Li *et al.*, 1996; Mertens *et al.*, 1996) and p38 δ (Jiang *et al.*, 1997; Wang *et al.*, 1997)

Ultraviolet light, mechanical stress, hyper-osmotic shock, hypoxia/reoxygenation, reactive oxygen species, pro-inflammatory cytokines such as interleukin-1 and tumour necrosis factor alpha (TNF- α), and to a lesser extent growth factors (Sugden and Clerk, 1998; Bogoyevitch, 2000), are some of the extracellular stresses that activate p38 MAPK during ischaemia and remain active during reperfusion (Pombo *et al.*, 1994; Bogoyevitch *et al.*, 1996; Kyriakis and Avruch, 1996). Activation of p38 has been implicated in transcription, protein synthesis, cell surface receptor expression and cytoskeleton integrity, ultimately affecting cell survival or leading to apoptosis and may even mediate cardiac hypertrophy (Obata *et al.*, 2000). Huot and co-workers (1997) reported that the p38 MAPK pathway may participate in several oxyradical-activated functions of the endothelium that are associated with reorganization of the microfilament network.

There is considerable controversy regarding the role of p38 in ischaemic injury and in preconditioning. Research has shown both protective and detrimental effects for p38 activation in ischaemia/reperfusion injury, where the p38 α isoform may be pro-apoptotic and the p38 β isoform may be anti-apoptotic (reviewed by Steenbergen, 2002).

2.6.4.1.1 Inhibition of p38 MAPK

A strong correlation between p38 activation and induction of apoptosis has been reported in cardiac myocytes exposed to ischaemia/reperfusion (Bogoyevitch *et al.*, 1996). Research has shown that inhibition of p38 MAPK activity during extended periods of ischaemia is cardioprotective (Mackay and Mochly-Rosen, 1999; Barancik *et al.*, 2000; Saurin *et al.*, 2000). In a cultured neonatal rat cardiac myocyte model, inhibition of p38 MAPK protects against ischaemic injury as evidenced by a reduction in LDH release (Mackay and Mochly-Rosen, 1999; Saurin *et al.*, 2000). Barancik and co-workers (2000) reported that the SB203580 inhibitor of p38 protected pig myocardium against ischaemic injury, suggesting that p38 activation may accelerate ischaemic cell death. Several studies indicated that p38 MAPK activation also plays a critical role in promoting myocardial apoptosis (Ma *et al.*, 1999; Mackay and Mochly-Rosen, 1999; Yue *et al.*, 2000). Ma and co-workers (1999) showed that p38 MAPK activity increased during ischaemia in isolated perfused rabbit heart, but administration of SB203580 before ischaemia and during reperfusion completely inhibited p38 MAPK activation, suggesting that inhibiting p38 was cardioprotective. Yue and co-workers (2000) also showed that SB203580 improved cardiac contractile function and offered cardioprotection in isolated ischaemic rat hearts by inhibiting p38 MAPK.

These studies show a correlation between inhibition of p38 MAPK activity and cardioprotection against ischaemia/reperfusion injury in myocytes as well as in isolated hearts.

2.6.4.1.2 Activation of p38 MAPK

On the other hand, research has also shown a protective role for p38 MAPK activation in the heart. Weinbrenner and co-workers (1997) demonstrated that tyrosine 182 phosphorylation of p38 MAPK was increased during a sustained period of ischaemia in preconditioned hearts compared to non-preconditioned hearts, which correlates with protection of preconditioning in the rabbit heart. Communal and co-workers (2000) reported that increased p38 MAPK activity together with decreased JNK1/2 activity, protects adult rat ventricular myocytes against beta-adrenergic receptor-stimulated apoptosis. Nakano and co-workers (2000) associated the protective effect of ischaemic preconditioning with the activation of MAPKAPK2 (a substrate of p38 MAPK) in the isolated rabbit heart. Zechner and co-workers (1998) reported myocardial protection from apoptosis with overexpression of MAPK kinase 6 (MKK6), an upstream activator of p38 MAPK. It appears that a distinct isoform of p38 MAPK, p38 β , participates in mediating cell survival (Wang *et al.*, 1998). Therefore, differential activation of p38 MAPK isoforms may exert opposing effects, where p38 α is implicated in cell death while p38 β may mediate myocardial survival. Although differential activation of p38 isoforms was observed in a study of simulated ischaemic injury in cultured neonatal cardiomyocytes (Saurin *et al.*, 2000), more research is needed on the effects of p38 isoforms in the intact adult myocardium. Differences in experimental protocol could also have contributed to these conflicting results of p38 isoforms.

2.6.4.2 c-Jun N-terminal kinases (JNK)

Although JNK phosphorylation appears to be pro-apoptotic in many cell types, their exact role in regulating cell death is unclear (Obata *et al.*, 2000; Park *et al.*, 2000). Wang and co-workers (1998) reported that activation of JNK by transfection of cultured rat neonatal cardiomyocytes with mitogen activated protein kinase (MKK7) induced hypertrophy rather than apoptosis, and co-activation of both JNK and p38 led to apoptosis.

The activation of JNK MAPKs has now been observed in a variety of cell types of the cardiovascular system. JNKs are important for cytokine biosynthesis and are involved in cell transformation and stress responses (Kyriakis and Avruch, 1996; Dong *et al.*, 1998; Kuan *et al.*, 1999). In almost all instances, the same stimuli that activate p38 also activate the JNKs (Kyriakis and Avruch, 1996). One exception is myocardial ischaemia/reperfusion where p38 is activated during ischaemia, and JNK during reperfusion (Pombo *et al.*, 1994; Bogoyevitch *et al.*, 1996; Kyriakis and Avruch, 1996). Furthermore, it has been reported by Laderoute and Webster (1997) that antioxidants inhibit the activation of JNKs in cultured myocytes subjected to hypoxia/reoxygenation.

2.6.4.2.1 The role of c-Jun N-terminal kinases (JNK) in apoptosis

Reoxygenation and reperfusion are known to activate JNK in the heart (Bogoyevitch *et al.*, 1996; Laderoute and Webster, 1997; Yue *et al.*, 1998; Davis, 2000), which is then translocated to the nucleus during ischaemia where it is phosphorylated during the reperfusion period (Pombo *et al.*, 1994; Yue *et al.*, 1998). Aoki and workers (2002) reported that the JNK pathway is a

direct activator of mitochondrial death machinery without other cellular components and provides a molecular linkage from oxidative stress to the mitochondrial apoptosis machinery. Lin (2003) proposed that JNK might be a modulator rather than an intrinsic component of the apoptotic machinery. Thus, JNK facilitates but does not induce apoptosis. These authors hypothesized that activated JNK inactivates suppressors of the apoptotic machinery. According to their model, JNK activation contributes to apoptosis only if the apoptotic process has already been activated.

In conclusion, both JNK and p38 are activated by ischaemia/reperfusion in the intact heart, but neither has been shown definitely to be pro- or anti-apoptotic.

2.6.4.3 Extracellular signal-regulated kinases (ERK)

Extracellular signal-regulated kinase-1 (ERK 1) and ERK 2, also known as p44 and p42 MAPKs, represent the prototypical MAPKs in mammalian cells. A wide variety of growth-promoting or hypertrophic agents activate these kinases in cardiac myocytes, fibroblasts, smooth muscle cells and endothelial cells (Bogoyevitch, 2000). Recently it has been shown that ERK activation also takes place in response to mitochondria-derived superoxide production secondary to the mitochondrial K_{ATP} opening (Samavati *et al.*, 2002).

2.6.4.3.1 The role of extracellular signal-regulated kinases (ERK) in apoptosis

The ERK pathway is important for survival of cells by protecting them from programmed cell death caused by stress-induced activation of JNK and p38 (Yue *et al.*, 2000).

Research has shown that inhibition of ERK enhances ischaemia/reperfusion-induced apoptosis and that sustained activation of this kinase during simulated ischaemia mediates adaptive cytoprotection in cultured neonatal cardiomyocytes (Punn *et al.*, 2000; Yue *et al.*, 2000). Omura and co-workers (1999) reported that both p42/44-MAPK and JNK were activated in the isolated Langendorff perfused rat heart exposed to global ischaemia/reperfusion, whereas in another experimental model of ischaemia/reperfusion in the intact heart, ERK1/2 activation was shown to attenuate the extent of apoptosis subsequent to reperfusion injury (Yue *et al.*, 2000).

Although research has shown that stress or agonist induced ERK1/2 activation is associated with protection of cardiac myocytes from apoptosis, little is known as how ERK signalling results in cellular protection. Cyclooxygenase-2 (COX-2) has been identified as a possible downstream mediator of protection in association with ERK 1/2 signalling in cardiomyocytes (Adderley *et al.*, 1999).

2.6.4.4 Protein kinase B (PKB/Akt) pathway

The serine/threonine protein kinase, protein kinase B or AKT (PKB/Akt), has emerged as a crucial regulator of cellular processes including apoptosis, proliferation and differentiation. PKB/Akt is activated downstream of PI-3-

kinase by the phosphoinositide-dependent protein kinases (PDK) PDK-1 and PDK-2 (Anderson *et al.*, 1998). PKB/Akt in turn phosphorylates a number of downstream targets relevant to cell survival function, including the pro-apoptotic Bcl-2 family member BAD (Del Peso *et al.*, 1997). Phosphorylation of BAD on Ser¹³⁶ by PKB/Akt inhibits its pro-apoptotic function, thus promoting cell survival (Datta *et al.*, 1997).

PKB/Akt has been suggested to be involved in cell survival pathways. Fujio and co-workers (2000) showed that PKB/Akt promotes survival of cardiomyocytes *in vitro* and protects against ischaemia/reperfusion injury in the mouse heart. Dominant negative alleles of PKB/Akt reduce ability of growth factors and other stimuli to maintain cell survival whereas over-expression of wild type or activated PKB/Akt can rescue cells from apoptosis induced by various stress signals (Kauffmann-Zeh *et al.*, 1997; Kennedy *et al.*, 1997). The mechanisms involved in cell survival have only recently begun to emerge. One way in which PKB/Akt may promote cell survival, is through direct phosphorylation of transcription factors that control the expression of pro- and anti-apoptotic genes.

2.6.5 Caspases

Activation of caspases is associated with apoptotic cell death. Caspases have been grouped into an upstream (caspases -1,-2,-4,-5,-8,-9,-10) and a downstream (caspases -3,-6,-7) subgroup (Nicholson and Thornberry, 1997). All caspases are composed of a prodomain and an enzymatic region. Upstream caspases are characterized by long prodomains that appear to

contain essential regulatory proteins. However, downstream caspases sensitive to DEVD (aspartate-glutamine-valine-aspartate) oligopeptides (caspase-3 and caspase-7) normally lead to the lethal proteolytic breakdown of cellular target proteins. For activation, the caspase proform has to be cleaved into a large subunit and a small subunit within the enzymatic domain that finally reassociates to form a complex comprising of two small and two large subunits. Substrates for caspases comprise many different proteins including nuclear proteins, proteins involved in signal transduction, and cytoskeletal targets (Cardone, 1997; Kothakota *et al.*, 1997). Most of these protein substrates appear to be cleaved by caspase -3 and -7. Although many of the target proteins defined to date have a nuclear localization, apoptotic cell death does not depend on the presence of a cell nucleus, as the characteristic cytoplasmic features of apoptosis can be observed in anucleate cytoplasts (Jacobson *et al.*, 1994).

2.6.5.1 Mechanisms of caspase activation

Recent data suggest that activation of caspases may take place either within death receptor complexes of the cytoplasmic membrane or by a mitochondrion-dependent mechanism within the cytosol (Zou *et al.*, 1997).

2.6.5.2 Poly-(ADP-ribose) polymerase (PARP)

A variety of stimuli induce apoptosis associated with cleavage of PARP by caspases (Kaufmann, 1989; Kaufmann *et al.*, 1993). Cleavage and inactivation of PARP prevents energy depletion and induction of necrosis. However, acute and massive DNA damage induces hyper-activation of PARP leading to

necrosis. Therefore, PARP cleavage has a function in the prevention of necrotic cell death that would otherwise lead to pathological inflammatory responses (Earnshaw, 1995).

Reperfusion injury is associated with caspase-3 activation, which results in cleavage of PARP to its proteolysed products (Engelbrecht *et al.*, 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Animal Care

All animals received humane care in accordance with the Principle of Laboratory Animal Care of the National Society of Medical Research and the Guide for the Care and use of Laboratory Animals of the National Academy of Sciences. (National Institutes of Health Publications no. 80-23, revised 1978).

3.2 Experimental Model 1

3.2.1 Experimental groups

Male Long-Evans rats were divided into two groups: a control group receiving standard rat chow and an experimental group receiving standard rat chow plus 7g RPO/kg diet for 6 weeks. The rat chow was supplied by Atlas Animal Foods, Cape Town, South Africa and regularly analyzed to monitor possible variations between batches. Red palm oil used for experimental work was supplied by the Malaysian Palm Oil Board. The approximate energy and macronutrient content of the two diets are indicated in Table 3.1.

The rats consumed an average of 25g food/day standard rat chow, containing 0.625g fat, which provides 8,7% of the energy intake. Protein intake was 4.5g (28% of energy intake). In the experimental group, 0.175g RPO baking fat was supplemented every morning, for 6 weeks, before they received their daily allowance of rat chow. Thus, there was a 21% increase in fat intake in the RPO-supplemented experimental group.

The red palm oil used in this study provided 70,0 µg carotenoids and 87,5 µg vitamin E (tocotrienols and tocopherols) additional to any other antioxidants

present in the standard rat chow diet (antioxidant nutrient status of standard rat chow diet not provided by suppliers due to confidentiality) (Nagadran *et al.*, 2000).

Table 3.1 Approximate energy and macronutrient content of rat diets

Nutrient	Standard rat chow diet (control) *	Standard rat chow diet supplemented with red palm oil **
Energy (kJ)	272,5	277,6
Total non-structural carbohydrates (g)	8,375	8,375
Total protein (g)	4,5	4,5
Total fat (g)	0,625	0,758
Total SFA (g)	0,139	0,206
Total MUFA (g)	0,168	0,219
Total PUFA (g)	0,297	0,312
Total n-6 PUFA (g)	0,247	0,248
Total n-3 PUFA (g)	0,049	0,061

* 25g of standard rat chow per day

** 25g of standard rat chow plus 0,175g red palm oil per day

SFA (g) = saturated fatty acids

MUFA (g) = monounsaturated fatty acids

PUFA (g) = polyunsaturated fatty acids

3.2.2 Working heart perfusion

At the end of the feeding programme, rats weighing 300-400g were anaesthetized with diethyl ether and intravenously injected with 400 units of heparin, before the hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer. Hearts were transferred to the standard working heart perfusion apparatus where they were perfused with a Krebs-Henseleit buffer

equilibrated with 95% O₂ and 5% CO₂ at 37 °C (118,5 mM NaCl; 4,75 mM KCl; 1,2 mM MgCl₂ · 6 H₂O; 1,36 mM CaCl₂; 25,0 mM NaHCO₃; 1,2 mM KH₂PO₄; 11,0 mM glucose) at a perfusion pressure of 100 cm H₂O.

The aorta was cannulated and retrograde perfusion with Krebs-Henseleit buffer was initiated. During this initial perfusion in the Langendorff mode, excess tissue was removed from the heart and the opening to the left atrium was cannulated.

Following a 5-minute stabilisation period in the Langendorff mode, hearts were switched to the working mode (Figure 3.1). The temperature of both the perfusate and the air surrounding the heart was thermostatically controlled and checked at regular intervals to ensure that the temperature was maintained at 37 °C irrespective of coronary flow. A cannula, connected to a pressure transducer, was inserted through the apex of the heart into the left ventricle. Left ventricular systolic and diastolic pressure, coronary flow (CF), heart rate (HR) and aortic output (AO) were measured at 25 minutes into perfusion. Hearts were then subjected to 25 minutes of global ischaemia. At the end of ischaemia, hearts were reperfused in the Langendorff mode for 10 minutes. In order to reduce the incidence of reperfusion arrhythmias, 2% lignocaine solution was used for the initial 3 minutes of reperfusion of all hearts. This was followed by a 15-minute working heart perfusion period during which cardiac function was measured. To assess fatty acid composition and NO-cGMP pathway activity, hearts were freeze-clamped with Wollenberger clamps pre-

cooled in liquid nitrogen, and freeze-dried to analyse for tissue cyclic nucleotide concentrations.

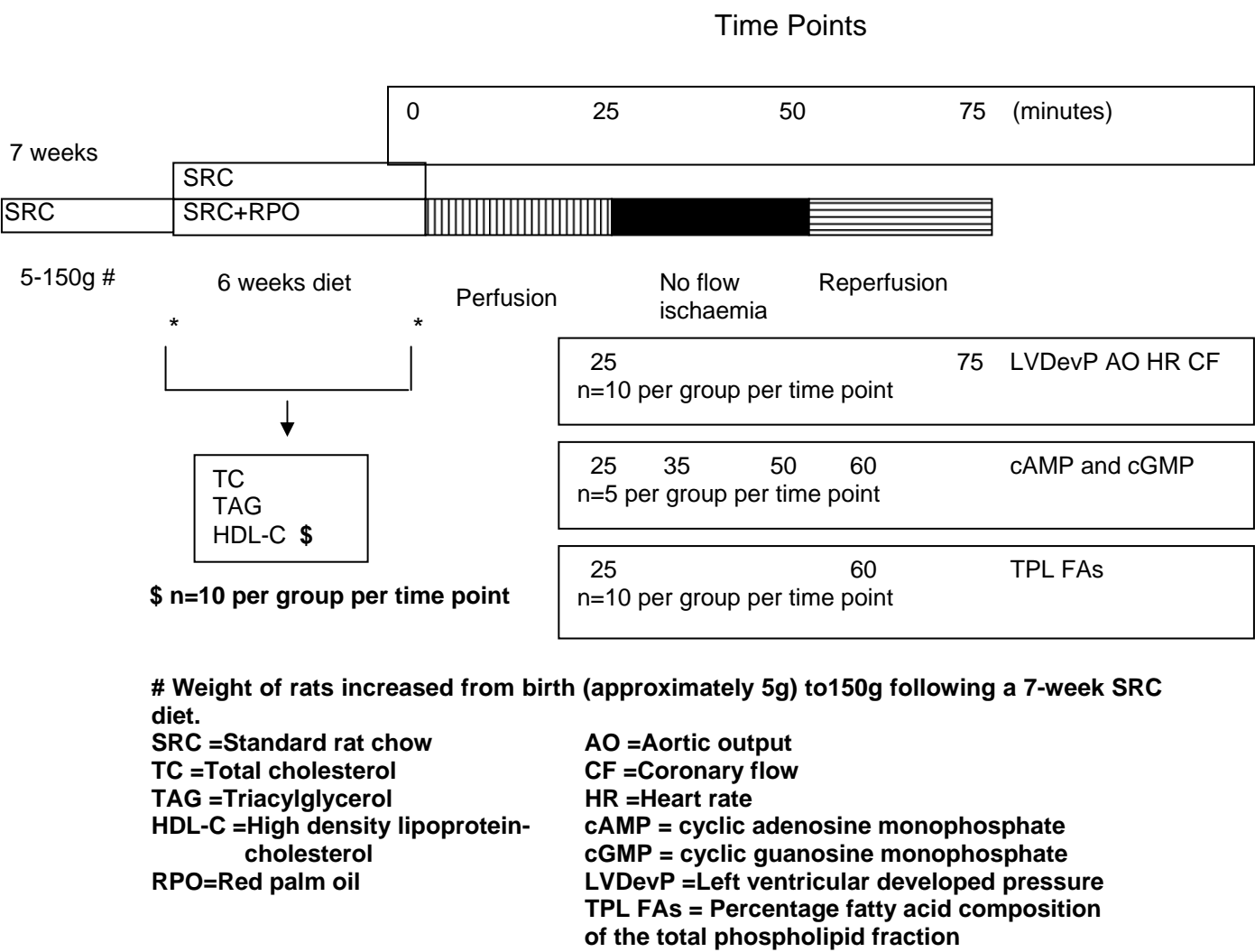


Figure 3.1 Study design for Experimental Model 1

3.2.3 Parameters measured and calculations used

3.2.3.1 Left ventricular developed pressure (LVDevP) (mmHg)

Left ventricular systolic-(LVSP) and diastolic (LVDP) pressure were monitored at 5-minute intervals during a 25-minute stabilization period and again during reperfusion after 25 minutes of global sustained ischaemia (n=10 per group per time point). LVDevP (the difference between systolic and diastolic pressure)

was used to measure mechanical function of the heart. It was calculated by comparing the LVDevP before and after ischaemia.

3.2.3.2 Aortic output recovery (AO)(%)

In order to compare the functional recovery of the hearts in the different groups, the heart rate (beats/min), coronary flow (ml/min) and aortic flow (ml/min) were measured by collecting 1-minute samples of the respective effluent (n=10 per group per time point). Aortic output (AO) recovery was calculated by dividing the AO after ischaemia by AO before ischaemia and expressing these values as a percentage recovery (see Figure 3.1 for time points used).

3.2.3.3 Biochemical analyses

The cAMP and cGMP concentrations were determined at regular intervals (see Figure 3.1) using radioimmunoassay kits obtained from Amersham Corp. (Amersham, UK).

3.2.3.3.1 cGMP assay

The assay is based on the competition between unlabelled cGMP and a fixed quantity of ¹²⁵I-labeled cGMP for a limited number of binding sites on a cGMP-specific antibody. Measurement of the radioactivity in the pellet enables the amount of labelled cGMP in the bound fraction to be calculated. The concentration of unlabelled cGMP in the sample is then determined by interpolation from a standard curve.

For cGMP assays freeze-clamped hearts (n=5 per group per time point, see Figure 3.1) were freeze-dried and 20-25 mg of dry tissue was extracted in 5% trichloroacetic acid. The extracted sample was ether-washed 3 times during 5-minute wash cycles. These samples were diluted 1:10 (V/V) with assay buffer and acetylated for the 125 I-labeled cGMP assay. The IC₅₀ for the cGMP assay was 25 pmol/tube (Du Toit *et al.*, 1999).

3.2.3.3.2 cAMP assay

The assay is based on the competition between unlabelled cAMP and a fixed quantity of the tritium labelled compound for binding to a protein which has a high specificity and affinity for cAMP. The amount of labelled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated. Separation of the protein bound cAMP from the unbound nucleotide is achieved by adsorption of the free nucleotide on to coated charcoal, followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

For the cAMP assays, 20-25 mg freeze-dried tissue was extracted with perchloric acid, neutralized and assayed (n=5 per group per time point, see Figure 3.1). The IC₅₀ for this assay was 1,92 mmol/tube (Du Toit *et al.*, 1999).

3.2.3.4 Heart muscle total phospholipid fatty acid composition (%)

Hearts isolated from rats fed standard rat chow or standard rat chow and 7g RPO per kg food for 6 weeks were perfused, freeze clamped and freeze-dried. The freeze-dried tissue was used to determine myocardial total phospholipid fatty acid composition (n=10 per group per time point). Tissue samples were extracted with chloroform: methanol (2:1; v/v) according to a modified method of Folch and co-workers (1957). Neutral lipids were separated from total phospholipids by thin-layer chromatography and the total phospholipid fraction analysed for fatty acid composition by gas chromatography. A fatty acid mixture was prepared from individual fatty acids (Sigma. St. Louis, MO, USA) and used as a standard. (Smuts *et al.*, 1992; Van Jaarsveld *et al.*, 2000).

3.2.3.5 Serum lipids

Rats were weighed weekly during the RPO-supplementation period and blood was collected from the tail vein before and after the 6-week period for both the standard rat chow (control) and the RPO-supplemented groups (n=10 per group per time point). The blood was centrifuged for 10 minutes at 3 000x g to obtain serum and analysed for serum lipid profiles i.e. total cholesterol (TC), high-density lipoprotein (HDL)-cholesterol and triacylglycerol. The serum lipid profile was determined using a Beckman Synchron Cx 9-instrument and Beckman Synchron Cx reagents (Beckman Coulter) employing enzymatic and colorimetric methods.

3.2.3.6 Statistical methods

Values are presented as mean \pm SEM. Some values are presented as percentage change from the baseline values. To calculate the percentage

change, the values were divided by baseline values and multiplied by 100. To adjust the X-axis to zero, 100 were subtracted from the values obtained. For paired comparisons the Student's t test was used. $P < 0,05$ was considered statistically significant.

3.3 Experimental Model 2

3.3.1 Experimental groups

Seven-week old Long-Evans rats were randomly allocated to 4 groups according to the dietary supplementation they received.

Group 1: Standard rat chow.

Group 2: Standard rat chow plus RPO (7 g RPO per kg diet).

Group 3: Standard rat chow, containing 2% cholesterol.

Group 4: Standard rat chow containing 2% cholesterol plus RPO (7 g RPO per kg diet).

The rats were fed a standard rat chow diet or 2% cholesterol-enriched diet (based on previous studies by Giricz *et al.*, 2003) for 6 weeks. Rats of the control group consumed an average of 25g food/day standard rat chow, containing 0.625g fat, which provides 8,7% of the energy intake. Protein intake was 4,5g (28% of energy intake). In two additional groups RPO-baking fat (7g RPO per kg diet) was supplemented every morning for 6 weeks to cholesterol-enriched and standard rat chow diet, respectively, before they received their daily allowance of rat chow. Thus, there was a 21% increase in fat intake in the RPO-supplemented experimental groups. The red palm oil used in this study provided 70,0 µg carotenoids and 87,5 µg vitamin E (tocotrienols and tocopherols) additional to that present in the standard rat chow diet (antioxidant nutrient status of standard rat chow diet not provided by supplier due to confidentiality) (Nagadran *et al.*, 2000).

3.3.2 Working heart perfusion and study design

The same protocol and study design (Figure 3.1 p.53) were used as discussed in Experimental Model 1 (see working heart perfusion 3.2.2 p.51).

3.3.3 Parameters measured and calculations used

The same measurements and calculations were used as discussed under paragraph 3.2.3 p.53 in Experimental Model 1. In addition, rate pressure product recovery was calculated for this study.

3.3.3.1 Rate pressure product recovery (RPP)(%)

Left ventricular developed pressure (LVDevP) was used to assess mechanical function of the heart (n=10 per group per time point). LVDevP recovery (%) = LVDevP 25 minutes into reperfusion (post-ischaemic)/ LVDevP 25 minutes into perfusion (pre-ischaemic) x 100.

Functional recovery was expressed as the percentage rate pressure product (RPP) recovery using the following formulae:

$$\text{RPP} = \text{Heart Rate (HR)} \times \text{LVDevP}$$

$$\% \text{ RPP recovery} = (\text{RPP reperfusion} / \text{RPP pre-ischaemic}) \times 100$$

3.3.3.2 Statistical Methods

Statistical methods were discussed in 3.2.3.6 p.56 (Experimental Model 1).

3.4 Experimental Model 3

3.4.1 Experimental groups

Four groups with the same dietary regimens as described in 3.3.1 p.58 (Experimental Model 2) were used. Male Wistar rats were used as experimental animals.

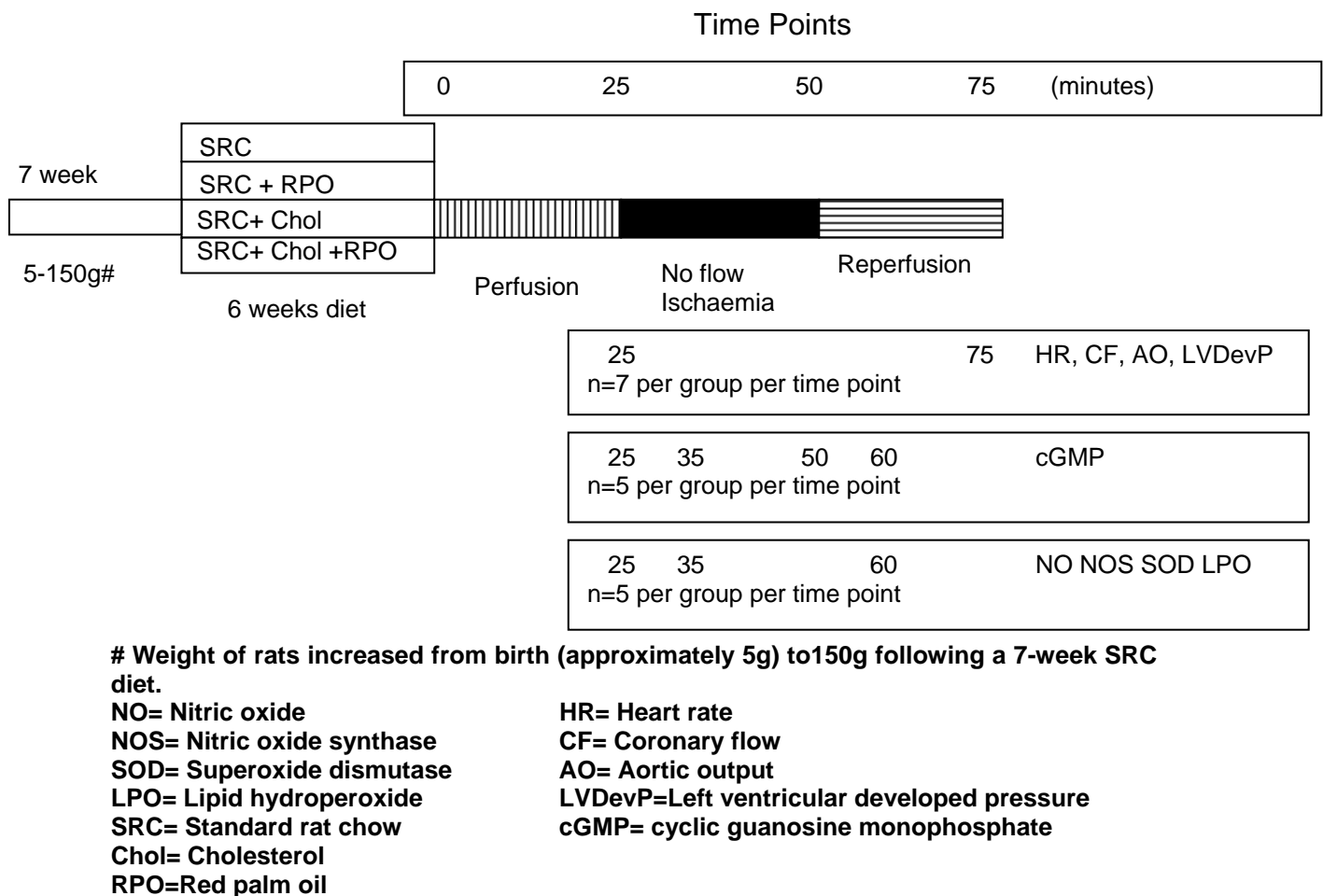


Figure 3.2 Study design for Experimental Model 3

3.4.2 Working heart perfusion

The protocol followed has been discussed in detail in 3.2.2 p.51 (Experimental Model 1). However, for assessment of myocardial nitric oxide concentrations, nitric oxide synthase- and superoxide dismutase activities and lipid hydroperoxide production, hearts were freeze-clamped with Wollenberger clamps pre-cooled in liquid nitrogen at the following times: at the end of the

pre-ischaemic working heart perfusion, after 10 minutes ischaemia and 10 minutes into reperfusion. Samples were stored at -80 °C for biochemical analyses (Figure 3.2 p.60).

3.4.3 Parameters measured and calculations used

3.4.3.1 Cardiac functional parameters and aortic output recovery (AO)(%)

Measurement of cardiac functional parameters and calculation of aortic output recovery have been discussed in section 3.2.3 p.53 under Materials and Methods in Chapter 3 (Experimental Model 1).

3.4.3.2 Measurement of cGMP

Determination of cGMP concentrations has been discussed in 3.2.3.3 p.54 (Experimental Model 1).

3.4.3.3 Measurement of cardiac nitric oxide concentrations

Approximately 200 mg of cardiac tissue was homogenized in 0,5 ml PBS (pH 7,4) and centrifuged at 10 000 x g for 20 minutes. The supernatant was ultra-filtered using a 30 kDa molecular weight cut-off filter (Millipore) and 40 µl of the filtrate was assayed.

Myocardial NO concentrations were determined using a Nitrate/Nitrite kit (Cayman Chemicals, Cayman Islands) which provides an accurate and convenient method for measurement of total nitrate/nitrite concentration in a simple two-step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of the Griess Reagents, which convert nitrite into a deep purple azo compound. Photometric

measurement of the absorbance due to this chromophore accurately determines nitrite concentration.

For direct measurement of intracellular NO, cardiomyocytes from RPO-supplemented and control hearts were isolated by collagenase perfusion, followed by incubation in a Krebs-Henseleit buffer containing 2% bovine serum albumin in the presence of 10 μ M diaminofluorescein-2/diacetate (DAF-2/DA) ($-0,5 \times 10^6$ cells/ml). Following incubation and washing, intracellular fluorescence of DAF-triazol (DAF-2T, oxidized from DAF-DAF-2/DA) was analyzed by flow cytometry (Strijdom *et al.*, 2004).

3.4.3.4 Measurement of cardiac nitric oxide synthase activity

Approximately 100 mg of cardiac tissue was homogenized in 0,5 ml homogenization buffer (1:10 dilution of stock solution, supplied by Cayman Chemicals) and centrifuged at 10 000 x g for 15 minutes at 4 °C. For the assay, 250 μ l of supernatant was used.

Total NOS activity was measured using a NOS assay-kit which is based on the biochemical conversion of L-arginine to L-citrulline by NOS (Cayman Chemicals, Cayman Islands). This reaction, which represents a novel enzymatic process, involves a 5-electron oxidation of guanidino nitrogen of L-arginine to NO, together with the stoichiometric production of L-citrulline. Advantages of this NOS kit include the use of radioactive substrate [14 C] arginine that enables sensitivity in the picomole range, as well as the specificity of the assay for the NOS pathway due to the direct enzymatic conversion of arginine to citrulline in eukaryotic cells. The NOS activity is quantified by

counting the radioactivity in the eluate (i.e. the flowthrough after reaction samples were added to spin cups and centrifuged for 30 seconds) after performing the assay.

3.4.3.5 Measurement of cardiac superoxide dismutase activity

Approximately 200 mg of cardiac tissue was homogenized in 1,0 ml ice-cold HEPES buffer and centrifuged at $1\,500 \times g$ for 5 minutes at 4 °C. For the assay, 10 µl of the supernatant was used.

Total activity of SOD was measured using a SOD kit (Cayman Chemicals, Cayman Islands) that utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthase. One unit of SOD is defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radical. The SOD assay measures all three types of SOD.

3.4.3.6 Measurement of cardiac lipid hydroperoxide production

Approximately 200 mg of cardiac tissue was homogenized in 0,5 ml HPLC-grade water. An equal volume of Extract R saturated methanol (Cayman Chemicals, Cayman Islands) was added and mixed thoroughly by vortexing. Cold chloroform (1,0 ml) was then added, mixed thoroughly and centrifuged at $1\,500 \times g$ for 5 minutes at 0 °C. The bottom chloroform layer was collected and 500 µl of this extract was used for the assay.

Lipid hydroperoxide (LPO) production in cardiac muscle was assessed using a LPO assay kit (Cayman Chemicals, Cayman Islands) which measures the hydroperoxides directly utilizing the redox reaction with ferrous ions.

Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thiocyanate ion as the chromagen.

3.4.3.7 Statistical Methods

Statistical methods were discussed in 3.2.3.6 p.56 (Experimental Model 1).

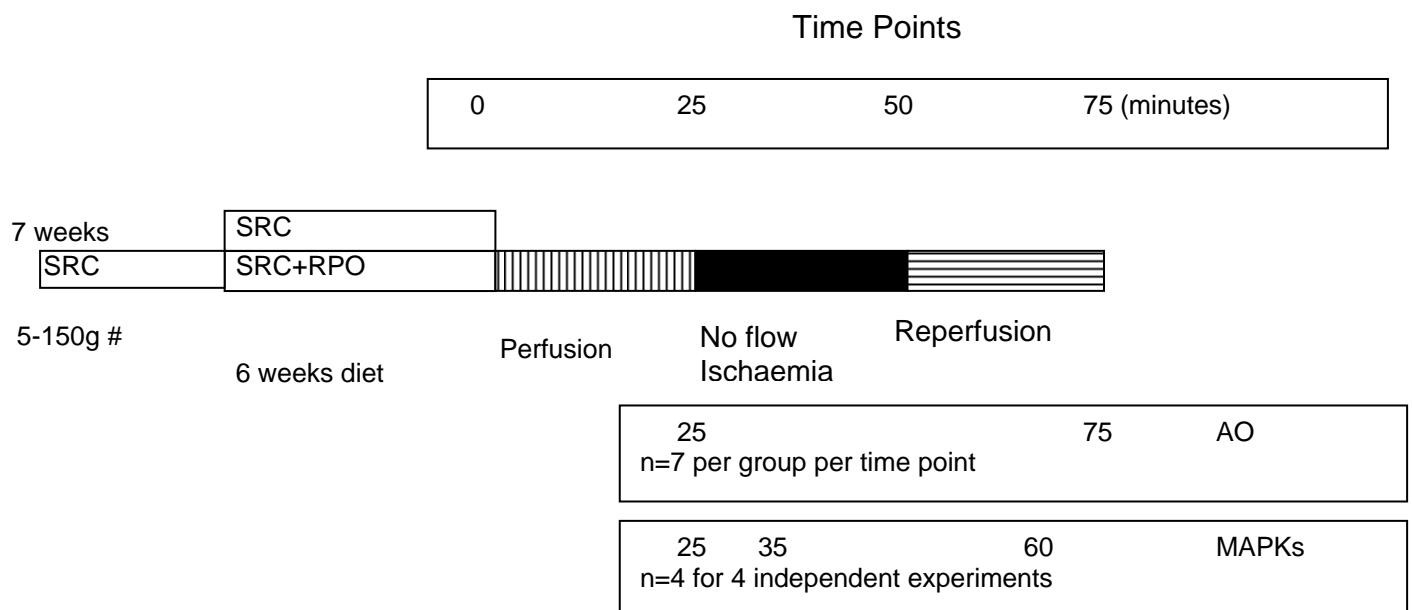
3.5 Experimental Model 4

3.5.1 Experimental groups

Two groups with the same dietary regimens as described in 3.2.1 p.50 were used. Experimental design is given in Figure 3.3. Male Wistar rats were used as experimental animals.

3.5.2 Working heart perfusion

Protocol followed has been discussed in detail in 3.2.2 p.51 (Experimental Model 1). To assess the activity of myocardial MAPKs, hearts were freeze-clamped with Wollenberger clamps pre-cooled in liquid nitrogen at the end of the pre-ischaemic working heart perfusion after 10 minutes ischaemia and 10 minutes into reperfusion and samples were stored at -80 °C (Figure 3.3).



Weight of rats increased from birth (approximately 5g) to 150g following a 7-week SRC diet.

SRC= Standard rat chow

RPO= Red palm oil

AO= Aortic output

MAPKs= Mitogen-activated protein kinases

Figure 3.3 Study design for Experimental Model 4

3.5.3 Parameters measured and calculations used

3.5.3.1 Aortic output recovery (AO)(%)

Measurement of AO has been discussed in 3.2.3.2 p.54.

3.5.3.2 Western blot analysis

Cardiac MAPKs and PKB/Akt, as well as caspase-3 and poly (ADP-ribose) polymerase (PARP) protein, were extracted with a lysis buffer containing: Tris, 20 mM; p-nitrophenylphosphate, 20 mM; EGTA, 1mM; sodium fluoride (NaF), 50 mM; sodium orthovanadate, 0.1 mM; phenylmethyl sulphonyl fluoride (PMSF), 1 mM; dithiothreitol (DTT), 1 mM; aprotinin, 10 µg/ml; leupeptin, 10 µg/ml. Mitogen-activated protein kinase phosphatase-1 (MKP-1) protein was extracted with a lysis buffer containing: Hepes, 50 mM; EDTA, 10 mM; EGTA, 10 mM; PMSF, 1 mM; Aprotinin, 1µg/ml; Leupeptin, 1µg/ml; Triton, 0,5%. The tissue lysates were diluted in Laemmli sample buffer and boiled for 5 minutes after the lysate protein content was determined using the Bradford technique (Bradford, 1976). For MAPKs and PKB/Akt 10µg protein, and 50µg protein for caspase-3 and PARP, were separated by 10% PAGE-SDS-gel electrophoresis. The separated proteins were transferred to a PVDF membrane (Immobilon™ P, Millipore). These membranes were routinely stained with Ponceau Red for visualization of proteins. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline, 0.1% Tween 20 (TBST) and then incubated with the primary antibodies that recognize phosphospecific ERK p42/p44 (Thr²⁰²/Tyr²⁰⁴), p38-MAPK (Thr¹⁸⁰/Tyr¹⁸²), JNK p54/p46 (Thr¹⁸³/Tyr¹⁸⁵), PKB (Ser⁴⁷³ and Thr³⁰⁸), caspase-3 (fragment pAb) and PARP (fragment pAb). Membranes were subsequently

washed with large volumes of TBST 5 x 5-minutes and the immobilized antibody conjugated with a diluted horseradish peroxidase-labelled secondary antibody (Amersham Life Sciences). After thorough washing with TBST, membranes were covered with ECLTM detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through a non-radioactive method (ECLTM Western blotting). Films were densitometrically analysed (UN-SCAN-IT, Silksience) and phosphorylated protein values were corrected for minor differences in protein loading, if required. Experiments were performed (data not shown) to ensure that all signals were within the linear range of detection on the autoradiographs under our assay and gel loading conditions.

Antibodies were purchased from Cell Signalling Technology and all other chemicals were obtained from Sigma (St Louis, MO).

3.5.3.3 Statistical methods

Data are presented as mean \pm SEM. Statistical significance was determined in multiple comparisons among independent groups of data in which ANOVA and the Bonferroni's post-hoc test indicated the presence of significant differences. A *P*-value < 0.05 was considered statistically significant.

CHAPTER 4

Dietary red palm oil supplementation protects against the consequences of global ischaemia in the isolated perfused rat heart.

Asia Pacific Journal of Clinical Nutrition (accepted 2004)

4.1 Abstract

Introduction: Activation of the NO-cGMP pathway is associated with myocardial protection against ischaemia. During ischaemia the function of this pathway is disturbed. Little is known about the effects of supplements such as red palm oil (RPO) on the myocardial NO-cGMP-signalling pathway. RPO consists of saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids and is rich in natural β -carotene and Vitamin E (tocopherols and tocotrienols). **Aims:** To determine whether dietary RPO-supplementation protects against the consequences of ischaemia/reperfusion and to identify a possible mechanism for this protection. **Methods:** Long-Evans rats were fed a standard rat chow (control) diet or standard rat chow diet plus 7g RPO per kg diet for 6 weeks. Hearts were excised and mounted on a working heart perfusion apparatus. Cardiac function was measured before and after hearts were subjected to 25 minutes of global ischaemia. Left ventricular systolic (LVSP) and diastolic pressure (LVDP), coronary flow (CF), heart rate (HR) and aortic output (AO) were measured. To assess NO-cGMP pathway activity, hearts subjected to the same conditions, were freeze-clamped and analysed for tissue cAMP and cGMP concentrations using a RIA method. Furthermore, composition of myocardial total phospholipid fatty acids was analysed by gas chromatography and blood samples were collected for serum lipid determinations. **Results:** The percentage aortic output recovery of hearts of the group supplemented with RPO was $72,9 \pm 3,5\%$ *versus* $55,4 \pm 2,5\%$ for controls ($P<0,05$). Ten minutes into ischaemia the cGMP concentrations of the RPO-supplementation group were significantly higher than the control group

($26,5 \pm 2,8$ pmol/g *versus* $10,1 \pm 1,8$ pmol/g). Myocardial total phospholipid PUFA content in the group supplemented with RPO increased from $54,5 \pm 1,1\%$ before ischaemia to $59,0 \pm 0,3\%$ after ischaemia ($P < 0,05$). **Conclusions:** Our results demonstrate that dietary RPO-supplementation protected hearts against ischaemia/reperfusion injury. These findings suggest that dietary RPO protect via the NO-cGMP pathway and/or changes in phospholipid PUFA composition during ischaemia/reperfusion.

4.2 Introduction

Palm oil and its liquid fraction, palm olein, are consumed worldwide as cooking oils and as constituents of margarines. These oils are also incorporated into fat blends used in the manufacturing of a variety of food products and in home food preparation. It plays a useful role in meeting energy needs and contributing to essential fatty acid (C18: 2n-6) needs in many regions of the world (Cottrell, 1991). Red palm oil (RPO) used in this study, contains a mixture of SFAs (51%), MUFAs (38%) and PUFAs (11%) (Nagendran *et al.*, 2000). Several clinical trials have evaluated palm oil's effects on blood lipids and lipoproteins. These studies suggest that palm oil and palm olein diets do not raise serum total cholesterol (TC) and LDL cholesterol concentrations to the extent expected from their fatty acid composition when a moderate-fat, moderate-cholesterol diet is consumed (Theriault *et al.*, 1999; Kritchevsky, 2000; Sundram and Basiron).

Although many animal feeding studies have shown that fish oil diets rich in n-3 PUFAs prevent ischaemia-induced cardiac arrhythmias (Nair *et al.*, 1997; Kang

and Leaf, 2000; Jump, 2002), only a few reports have been published on the protective effects of RPO-supplementation against ischaemia/reperfusion injury (Abeywardena *et al.*, 1991; Charnock *et al.*, 1991; Abeywardena and Charnock, 1995).

Palm oil and palm oil products are also natural occurring sources of the antioxidant vitamin E constituents tocopherols and tocotrienols. These natural antioxidants act as scavengers of damaging oxygen free radicals. Two studies published in the New England Journal of Medicine show that both men and women who supplement their diet with at least 100 IU of vitamin E (67 μ g natural α -tocopherol) per day for at least two years have a 37-41% drop in the risk of heart disease (Rimm *et al.*, 1993; Stampfer *et al.*, 1993). Vitamin E is believed to be the major lipid-peroxidation chain-breaking antioxidant found in blood plasma and membranes. The insight into the mechanism of heart injury has suggested that administration of antioxidants may lessen oxidative damage of the heart. It has been shown that a palm oil vitamin E mixture containing both α -tocopherol and α -tocotrienol, was more efficient in the protection of the isolated Langendorff heart against ischaemia/reperfusion injury than tocopherol alone as measured by its mechanical recovery (Serbinova *et al.*, 1992). Palm oil vitamin E completely suppressed LDH enzyme leakage from ischaemic hearts, prevented the decrease in ATP and creatine phosphate levels, and inhibited the formation of endogenous lipid peroxidation products (Serbinova *et al.*, 1992).

Nitric Oxide (NO) is an important regulator of both cardiac and vascular function and tissue reperfusion (reviewed by Ferdinandy and Schultz, 2003). Myocardial NO formation is increased during ischaemia and reperfusion, offering protection against ischaemia/reperfusion injury (Maulik *et al.*, 1995; Williams *et al.*, 1995; Araka *et al.*, 2000). However, when NO plummets during reperfusion, due to rapid quenching by superoxide, the vascular protective regulatory properties become dysfunctional. This may lead to exacerbation of tissue injury. Reactive oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) have long been implicated in the pathogenesis of ischaemia/reperfusion injury. These radicals are predominant both during ischaemia and at the time of reperfusion, and can react with nucleic acids, proteins and lipids, resulting in damage to the cell membrane or intracellular organelles (Gilham *et al.*, 1997). Peroxynitrite ($ONOO^-$) is generated by a diffusion-limited reaction between O_2^- and NO (Rubbo *et al.*, 1994; Naseem *et al.*, 1995; Ferdinandy and Schultz, 2003).

Protective effects of NO are mediated through the production of cGMP. NO donors given during ischaemia possibly protect the myocardium by increasing tissue cGMP and decreasing cytosolic Ca^{2+} overload. Increases in cAMP concentrations associated with ischaemia would increase Ca^{2+} concentrations and exacerbate ischaemic and reperfusion injury (Du Toit *et al.*, 2001). In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP induced slow inward calcium current, thus leading to a decrease in cytosolic calcium concentrations (Sumii and Sperelakis, 1995).

Because little is known about the protective effect of RPO and the mechanisms involved, our aim was to determine whether dietary RPO-supplementation protects against the consequences of ischaemia and reperfusion.

4.3 Materials and methods

4.3.1 Experimental Model

Long-Evans rats were fed a standard rat chow (control) diet or standard rat chow diet plus 7g RPO per kg diet for 6 weeks. The working heart perfusion method used in this experimental model is described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50) and the protocol that was followed is summarized in Figure 3.1 p 53.

4.3.2 Measurement of cardiac function

Left ventricular systolic (LVSP) and diastolic pressure (LVDP), coronary flow (CF), heart rate (HR) and aortic output (AO) were measured as described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

4.3.3 Biochemical analyses

The cAMP and cGMP concentrations were determined using radioimmunoassay kits obtained from Amersham Corp. (Amersham, UK). The principle and methods were discussed in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

4.3.4 Heart muscle total phospholipid fatty acid composition (%)

Composition of myocardial total phospholipid fatty acids was analysed by gas chromatography as described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

4.3.5 Serum lipids

The serum lipid profile was determined using a Beckman Synchron Cx 9-instrument and Beckman Synchron Cx reagents (Beckman Coulter) as described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

4.3.6 Statistical methods

Statistical methods used were discussed in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

4.4 Results

4.4.1 Left ventricular developed pressure (LVDevP)

The pre-ischaemic LVDevP of hearts of RPO-supplemented and control groups were similar (Figure 4.1). After 25 minutes of global ischaemia and 25 minutes of reperfusion, the percentage LVDevP recovery of hearts from the RPO-supplemented group was $89,0 \pm 8,1\%$ as compared to the $81,0 \pm 6,0 \%$ LVDevP recovery of hearts obtained from rats fed the standard rat chow (control) diet (n=10 per group per time point).

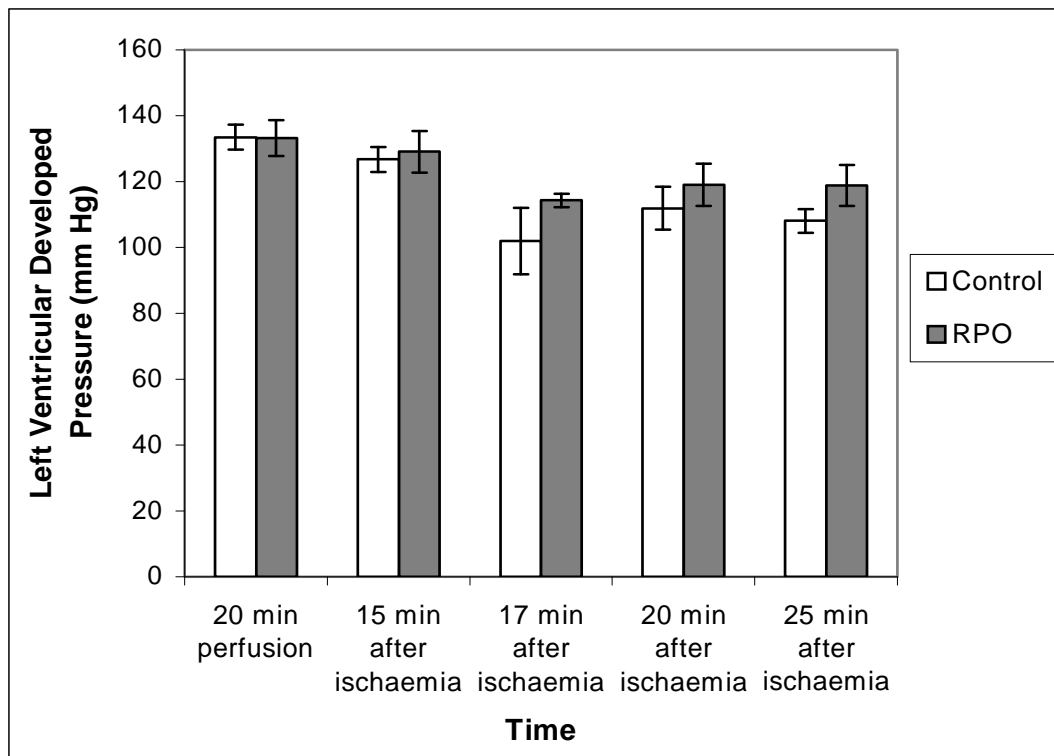


Figure 4.1 The effect of RPO-supplementation on left ventricular developed pressure during pre-ischaemic perfusion and reperfusion (n=10 per group per time point) (mean \pm SEM)

4.4.2 Aortic output recovery (%)

After 25 minutes of global ischaemia and 25 minutes of reperfusion, percentage aortic output (AO) recovery of hearts from the RPO-supplemented group was $72,9 \pm 3,4\%$ *versus* $55,4 \pm 2,5\%$ for the control group ($P < 0,05$) (Figure 4.2) (n=10 per group per time point).

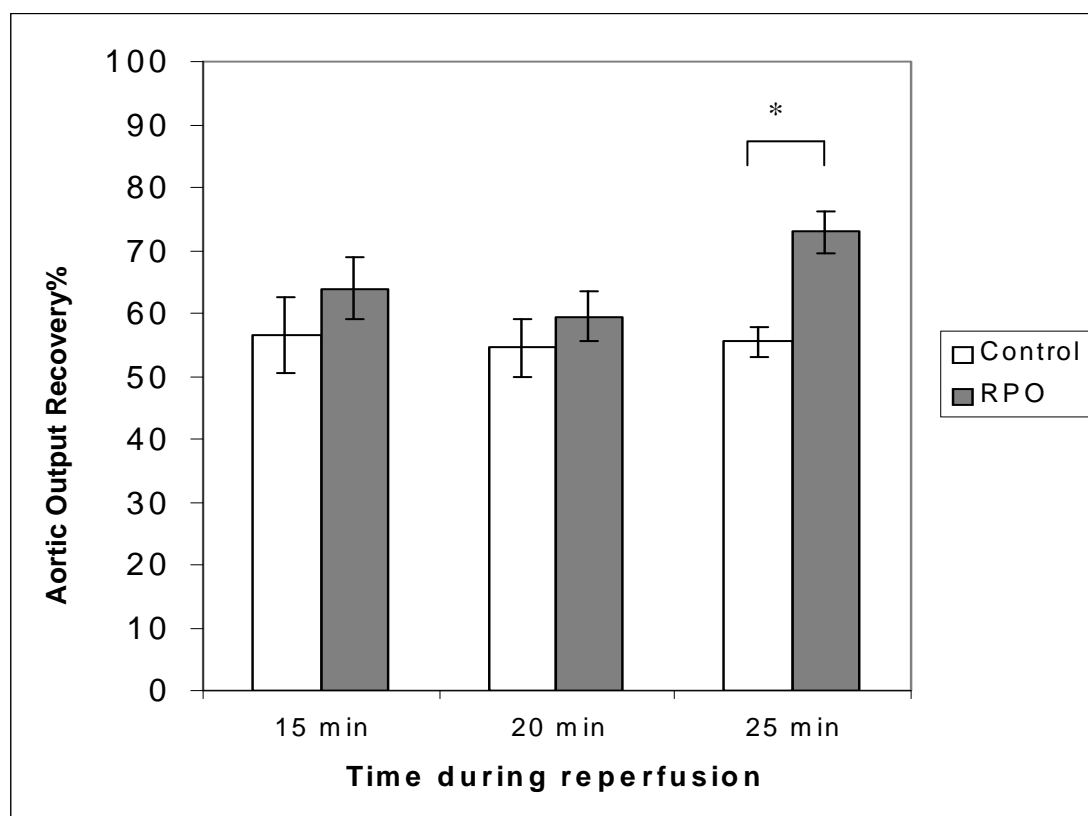


Figure 4.2 % Aortic output recovery of hearts in the RPO-supplemented group versus hearts of the control group (n=10 per group per time point) (* $P<0.05$) (mean \pm SEM)

4.4.3 Effects of RPO-supplementation on ischaemic cAMP and cGMP concentrations

Myocardial cAMP concentrations were not affected by RPO-supplementation (n=5 per group per time point) (Figure 4.3).

The cGMP concentrations at 10 minutes into ischaemia were $26,5 \pm 2,8$ pmol/g in hearts of the RPO-supplemented group, compared to $10,1 \pm 1,8$ pmol/g in hearts of the control group ($P<0,05$) (n=5 per group per time point) (Figure 4.4).

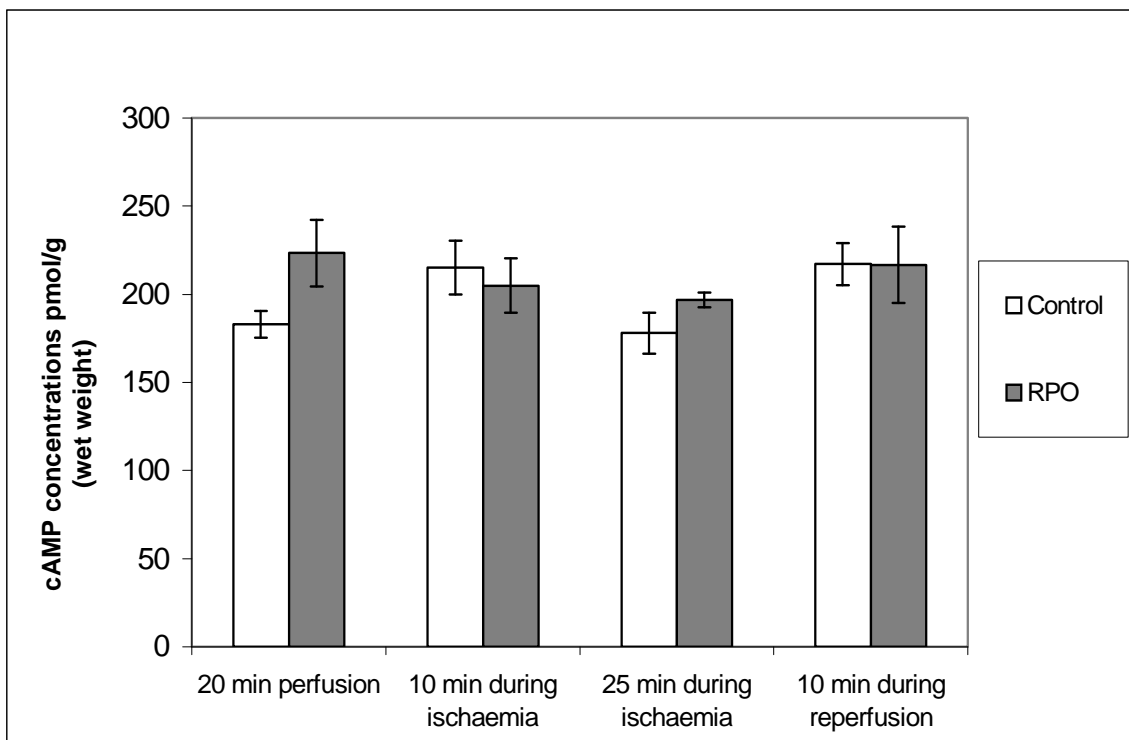


Figure 4.3 Myocardial cAMP concentrations for hearts of rats on RPO-supplemented diet *versus* hearts of control diet (n=5 per group per time point) (mean \pm SEM)

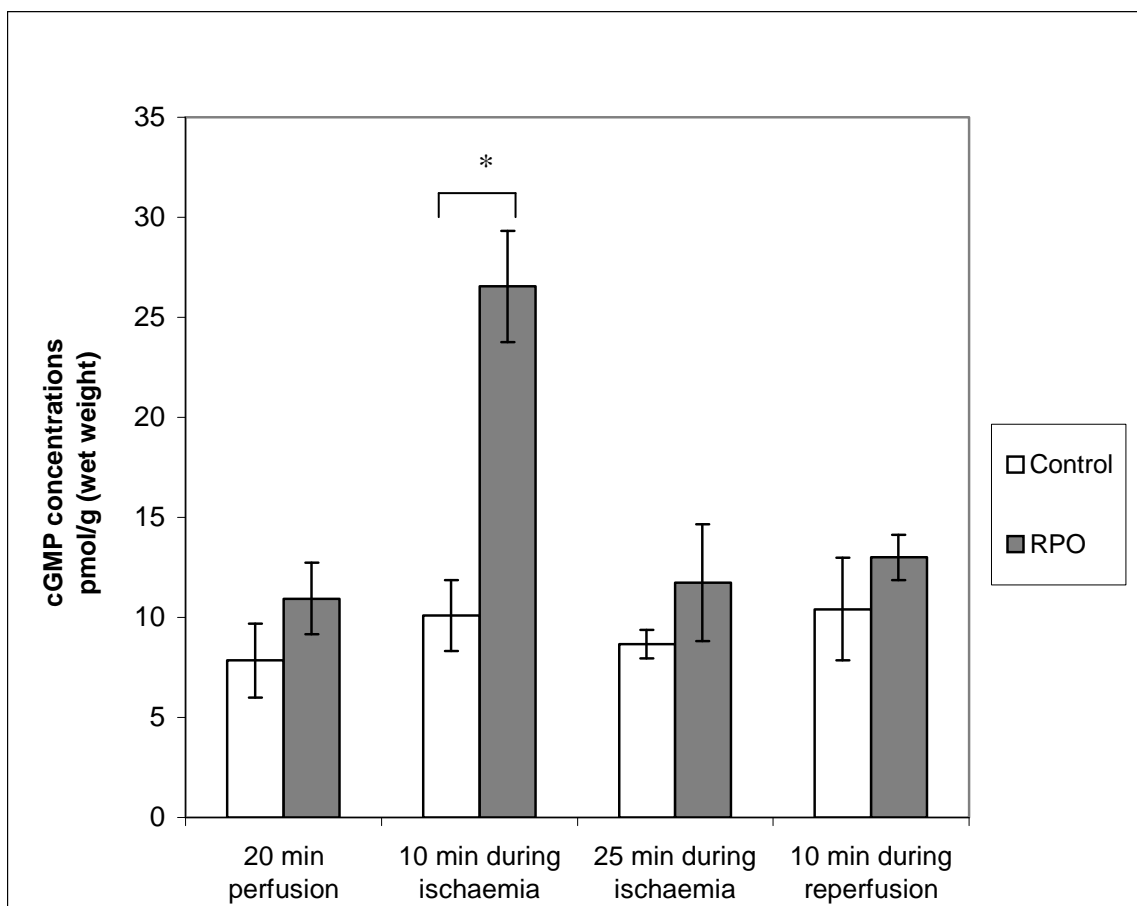


Figure 4.4 Myocardial cGMP concentrations for hearts of rats on RPO-supplemented diet *versus* hearts of control diet (n=5 per group per time point) (* $P < 0.05$) (mean \pm SEM)

4.4.4 Serum lipids

Weight increase of rats remained stable between the control group and RPO-supplemented group after the 6-week supplementation period.

All values in Figures 4.5 represent the percentage change in serum total cholesterol-, high density lipoprotein cholesterol and triacylglycerol concentrations between the baseline value and the value of the corresponding experimental group after the 6-week supplementation period (n=10 per group).

Although there were baseline differences between the control and RPO-supplemented groups ($1,2 \pm 0,04$ mmol/l *versus* $1,4 \pm 0,04$ mmol/l, $P < 0,05$), the % change in serum total cholesterol showed no difference between the two experimental groups after the 6-week diet period (n=10 per group).

HDL-cholesterol followed a similar trend with baseline differences between control and RPO-supplemented groups ($1,0 \pm 0,03$ mmol/l *versus* $1,2 \pm 0,05$ mmol/l, $P < 0,05$), but the % change in HDL-cholesterol showed no difference between the two groups after the 6-week diet period (n=10 per group) (Figure 4.5).

Serum concentrations of triacylglycerol were significantly increased in the RPO-supplemented group after 6 weeks on the diet (before: $0,6 \pm 0,1$ mmol/l *versus* after: $0,9 \pm 0,1$ mmol/l, $P < 0,05$) (n=10 per group) (Figure 4.5). However, percentage change in serum triacylglycerol between the control- and RPO-supplemented groups showed no difference.

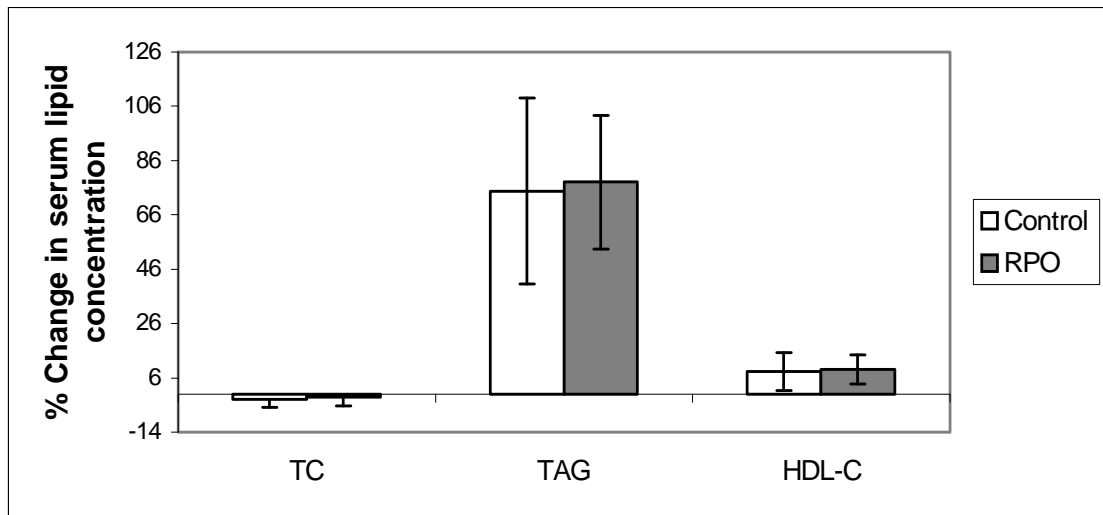


Figure 4.5 % Change in serum lipid concentration in control and RPO supplemented groups after 6 weeks (n=10 per group) (mean \pm SEM)

TC = Total cholesterol

TAG = Triacylglycerol

HDL-C = High density lipoprotein-cholesterol

4.4.5 Heart muscle total phospholipid fatty acid composition (%)

Myocardial total phospholipid fatty acid composition is presented in Figure 4.6.

Our results show that hearts of the group supplemented with RPO caused a significant increase in pre-ischaemic (baseline) myocardial total SFA composition ($38,0 \pm 1,0\%$) *versus* control hearts ($34,4 \pm 0,4\%$) ($P < 0,05$). No significant changes occurred in MUFA, PUFA, total n-6 and total n-3 composition between groups.

During the perfusion protocol, the event of ischaemia altered fatty acid composition. The myocardial total SFA composition decreased significantly from $38,0 \pm 1,0\%$ before ischaemia to $33,6 \pm 0,2\%$ after ischaemia ($P < 0,05$) in the hearts of the group supplemented with RPO.

Concurrently, myocardial total phospholipid PUFA composition in hearts of the group supplemented with RPO increased from $54,5 \pm 1,1\%$ before ischaemia

to $59,0 \pm 0,3\%$ after ischaemia ($P < 0,05$), with no changes in myocardial total phospholipid MUFA composition.

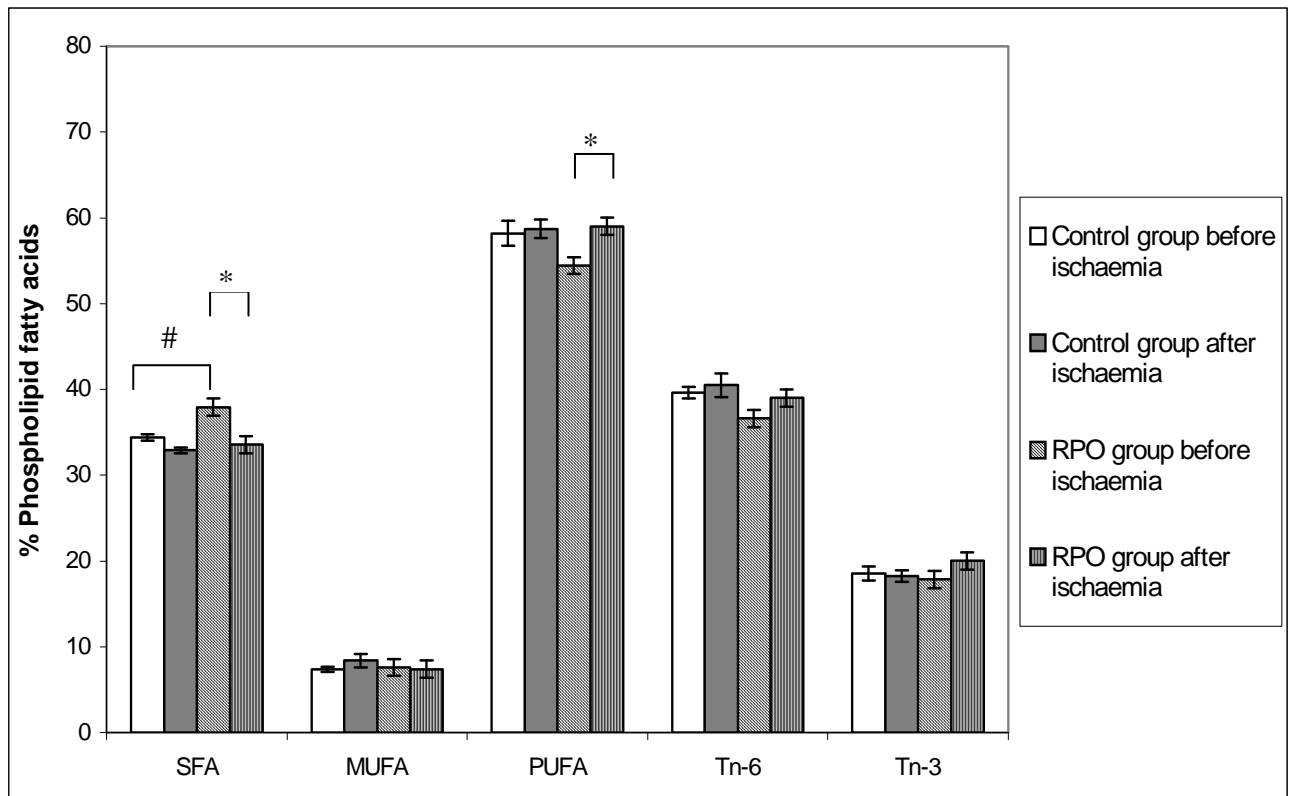


Figure 4.6 Heart muscle total phospholipid fatty acids 20 minutes in perfusion and 10 minutes in reperfusion respectively, for RPO supplemented group versus control group (n=10 per group) (* $P < 0,05$ for the group before and after ischaemia) (# $P < 0,05$ for RPO supplemented group versus the corresponding control group) (mean \pm SEM)

SFA= Total saturated fatty acids
 MUFA= Total monounsaturated fatty acids
 PUFA= Total polyunsaturated fatty acids
 Tn-6= Total n-6 polyunsaturated fatty acids
 Tn-3= Total n-3 polyunsaturated fatty acids

4.5 Discussion

Our results demonstrate that RPO-supplementation offered protection against ischaemia/reperfusion injury in the isolated perfused working heart as reflected by improved aortic output recovery. These data support the findings of Serbinova and co-workers (1992) who showed that palm oil vitamin E was more effective in the protection against ischaemia/reperfusion injury in the isolated Langendorff perfused heart than tocopherol alone.

Based on our results we propose that the protective effect of RPO may be associated with either its antioxidant characteristics and/or changes in the myocardial total phospholipid fatty acid composition during ischaemia/reperfusion. Abeywardena and co-workers (1991) also argued that RPO protection lies in an alliance of fatty acids and endogenous antioxidants during ischaemia/reperfusion.

Dietary RPO-supplementation for 6 weeks caused significant changes in heart muscle total phospholipid fatty acid composition. The decrease in total phospholipid SFA composition of the heart muscle was accompanied by an increase in the total phospholipid PUFA composition, which could be associated with improved reperfusion aortic output recovery. Normally, linoleic acid (LA) undergoes a series of elongations and desaturations to yield arachidonic acid (AA). To our knowledge no data exist on the effect of ischaemia on elongase and desaturase activity. Many studies have focused on the effect of FA on arrhythmias. Contradictory results exist on the effect of AA

on the development of arrhythmias. Li and co-workers (1997) found that free AA is able to prevent arrhythmias, but the major cyclooxygenase metabolites (PGD₂, PGE₂, PGF₂ and TXA₂) derived from AA are arrhythmogenic. However, prostacyclins (PGI₂), also synthesized from AA, are anti-thrombotic agents that act as a vasodilator in blood vessels and have an antiarrhythmogenic effect. No clear relationship exists between the availability of AA in myocardial phospholipids and eicosanoid profile. Abeywardena and co-workers (1991) showed that a chemically refined palm oil-supplementation for 12 months had no effect on prostacyclin production from AA in rat myocardial tissue. Concurrently, thromboxane A₂ production was inhibited. Another palm oil supplement (with a near-identical fatty acid profile) in the same study showed no thromboxane A₂ inhibition. This argues that thromboxane A₂ production is unlikely to be mediated via fatty acids. In another study (Abeywardena *et al.*, 1995) myocardial prostacyclin production was increased after ischaemia with refined, bleached and deodorized palm oil (RBD-PO)-supplementation for 9 months. However, these authors argue that this increase in prostacyclin production may not be mediated by fatty acids alone but that endogenous antioxidants may also play a role.

The increase in PUFAs during ischaemia/reperfusion in the current study, suggests that PUFAs may be involved in the protection against ischaemia/reperfusion injury. The mechanism of phospholipid fatty acid protection remains elusive and needs further investigation.

Generally, the mechanism of eicosanoid action is to bind to membrane receptors, leading to generation of second messengers such as cAMP and Ca^{2+} (Charnock *et al.*, 1991; Mohan *et al.*, 1995; Li *et al.*, 1997). As myocardial cAMP concentrations were not significantly affected throughout the protocol in the hearts of the group supplemented with RPO, prevention of fatal cardiac arrhythmias may depend on the net effect of these metabolites, which is determined by the status of AA metabolism and the contents of n-6 and other fatty acids (Abeywardena *et al.*, 1991; Charnock *et al.*, 1991; Mohan *et al.*, 1995; Li *et al.*, 1997).

Interaction between cardiac endothelium-derived prostaglandins and NO determines myocardial performance (Mohan *et al.*, 1995). Our results suggest that the NO-cGMP pathway is involved in the protective effect of RPO. The elevated cGMP concentrations early in ischaemia may suggest that RPO-supplementation protected the isolated rat heart against ischaemia/reperfusion injury via the NO-cGMP pathway. NO is known to increase myocardial cGMP and it can be speculated that the protective effect of NO is related to a mechanism secondary to the stimulation of guanylyl cyclase within the vascular wall or in ventricular myocytes (Beresewics *et al.*, 1995). Besides its effects on myocardial contractility, the NO-cGMP pathway stimulation during ischaemia may protect the heart against ischaemia/reperfusion-induced calcium overload. In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP-induced slow inward calcium current, thus leading to a decrease in cytosolic calcium levels (Du Toit *et al.*, 2001). Another protective pathway might include the NO-cGMP dependent pathway in which

the sarcolemmal- K_{ATP} channels are opened and the cystolic calcium concentrations are lowered (Du Toit *et al.*, 2001). Therefore, cGMP appears to be an endogenous intracellular cardioprotectant (Pabla *et al.*, 1995).

Studies have shown that reactive oxygen species (ROS) can oxidize lipids and proteins and contribute to myocardial injury (reviewed by Illarion *et al.*, 2002). Peroxynitrite ($ONOO^-$) is generated by a diffusion-limited reaction between O_2^- and NO (Naseen *et al.*, 1995; Ferdinandy *et al.*, 2003). Palm oil and palm oil products are naturally occurring sources of the antioxidant vitamin E constituents tocopherol and tocotrienols, which act as scavengers of these damaging oxygen free radicals which may lessen oxidative damage to the heart (Serbinova *et al.*, 1991). This may suggest that NO, synthesized from L-arginine by NO-synthase (NOS), results in enhanced synthesis of cGMP. NO synthase activity in the heart is rapidly stimulated by ischaemia and this stimulation is maintained during the whole ischaemic episode (Moncado and Higgs, 1993; Depré *et al.*, 1996). Our data show that RPO-supplementation increases cGMP concentrations that may confer some of the cardioprotection to the ischaemic and reperfused rat heart.

Although there were baseline differences between the control and RPO-supplemented groups, the serum total cholesterol concentration was not effected by the 6-week diet. These data are in agreement with other studies which showed that not all saturated dietary fats raise serum total cholesterol concentration (Theriault *et al.*, 1999; Kritchevsky, 2000; Sundram and Basiron). Serum triacylglycerols concentrations were increased in both groups

after the 6-week period with a significant difference within the RPO-supplemented group. The baseline difference in serum total cholesterol between the control and RPO-supplemented groups cannot be explained and requires further investigation, since rats were randomly allocated to groups.

Currently little documented data exist on the effects of dietary RPO-supplementation and post ischaemic recovery linked to the NO-cGMP pathway, heart muscle total phospholipid fatty acid composition and antioxidant status. The findings of this study create opportunities for further investigations to elucidate mechanisms involved in cardiac protection.

4.6 Conclusion

Dietary RPO-supplementation protects against the consequences of global ischaemia in the isolated perfused rat heart from animals on a standard rat chow (control) diet. The mechanism of protection may involve the NO-cGMP pathway and/or myocardial phospholipid fatty acid compositional changes during ischaemia/reperfusion.

Little knowledge exists on the protective effects of RPO-supplementation against ischaemia/reperfusion injury, especially when supplemented with a high-cholesterol diet. It was therefore our next aim to investigate whether RPO-supplementation offers the same protection against ischaemia/reperfusion injury when supplemented with a high-cholesterol diet, which simulates an unhealthy dietary intake.

CHAPTER 5

Dietary red palm oil improves reperfusion cardiac function in the isolated perfused rat heart of animals fed a high-cholesterol diet.

Prostaglandins, Leukotrienes and Essential Fatty Acids (published 2005)

5.1 Abstract

Introduction: It has been shown that dietary red palm oil (RPO)-supplementation of rats on a standard rat chow (SRC) diet improved reperfusion function. However, no exact cellular protective mechanisms have been established. **Aims:** The aims of this study were to determine whether dietary RPO-supplementation offers the same protection when cholesterol was added to the diet and if so, to determine a possible cellular mechanism and a role for fatty acids. **Methods:** Rats were fed a standard rat chow, standard rat chow plus cholesterol and/or RPO-supplementation for 6 weeks. Functional recovery, myocardial total phospholipid fatty acid composition and cAMP/cGMP concentrations were determined in isolated rat hearts subjected to 25 minutes of normothermic total global ischaemia. **Results:** Dietary RPO in the presence of cholesterol improved aortic output recovery ($63,2 \pm 3,1\%$) *versus* cholesterol only ($36,5 \pm 6,2\%$, $P<0,05$). The improved functional recovery of hearts from rats supplemented with RPO *versus* control group was preceded by an elevation in the cGMP concentrations early in ischaemia (RPO $132,9 \pm 36,3\%$ *versus* control $42,7 \pm 24,4\%$, $P<0,05$). Concurrently, cAMP concentrations decreased (RPO: $-8,3 \pm 6,9\%$ *versus* control: $19,9 \pm 7,7\%$, $P<0,05$). **Conclusions:** Our data suggest that dietary RPO-supplementation improved reperfusion aortic output through mechanisms that may include activation of the NO-cGMP and inhibition of the cAMP pathway.

5.2 Introduction

Red palm oil consists of 51% saturated fatty acids (SFAs), 38% monounsaturated fatty acids (MUFAs), 11% polyunsaturated fatty acids (PUFAs) and a spectrum of antioxidants with carotenoids, tocopherols and tocotrienols as the major constituents (Nagendran *et al.*, 2000). It has been shown by Serbinova and co-workers (1992) that a palm oil vitamin E mixture containing both α -tocopherol and α -tocotrienol, improved reperfusion functional recovery in a Langendorff perfused rat heart. They argued that the protection was induced by the ability of both α -tocopherols and α -tocotrienols to scavenge free radicals during reperfusion, but that α -tocotrienols offered more efficient protection. Apart from this finding little knowledge exist on the exact mechanism of dietary RPO-induced cardioprotection against ischaemia/reperfusion injury. The composition of RPO allows for protection to be offered by either the fatty acid constituents or the anti-oxidative carotenoids, tocopherols and tocotrienols (Abeywardena *et al.*, 1991; Charnock *et al.*, 1991; Cottrell, 1991; Abeywardena and Charnock, 1995; Chandrasekharan, 1999; Theriault *et al.*, 1999) or both. A possible mechanism that has not been investigated may include the myocardial NO-cGMP pathway.

Reactive oxygen species, including superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), have long been implicated in the pathogenesis of ischaemia/reperfusion injury. These radicals are generated both during ischaemia and at the time of reperfusion and can react with nucleic acids, proteins and lipids, resulting in damage to the cell membrane or

intracellular organelles (Gilham *et al.*, 1997). Peroxynitrite (ONOO^-) is generated by a diffusion-limited reaction between O_2^- and nitric oxide (NO) (Rubbo *et al.*, 1994; Naseen *et al.*, 1995; Ferdinandy *et al.*, 1999; Ferdinandy and Schultz, 2003).

NO is an important regulator of both cardiac and vascular function and tissue reperfusion (reviewed by Ferdinandy and Schultz, 2003). Myocardial NO formation is increased during ischaemia and reperfusion, offering protection against ischaemia/reperfusion injury (Williams *et al.*, 1995; Araki *et al.*, 2000; Bolli, 2001). Protective effects of NO are mediated through the production of cGMP. NO is known to increase myocardial cGMP, and it can be speculated that the protective effect of NO is related to a mechanism secondary to the stimulation of guanylyl cyclase within the vascular wall, or in ventricular myocytes (Maulik *et al.*, 1995; Beresewics *et al.*, 1995; Depré *et al.*, 1997). NO donors given during ischaemia possibly protect the myocardium by increasing tissue cGMP and decreasing cytosolic Ca^{2+} overload. Increases in cAMP concentrations associated with ischaemia would increase Ca^{2+} concentrations and exacerbate ischaemia/reperfusion injury (Du Toit *et al.*, 2001). In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP-induced increase in slow inward calcium current, thus leading to a decrease in cytosolic calcium concentrations (Sumii and Sperelakis, 1995). Therefore, cGMP appears to be an endogenous intracellular cardioprotectant (Pabla and Curtis, 1995; Pabla *et al.*, 1995).

Cardiac stress adaptation is possibly compromised in hyperlipidaemia due to altered NO-cGMP pathway function. Research showed that high-cholesterol diets impair NO-cGMP signalling in both endothelium and non-endothelial cells with a significant decrease in cardiac NO concentration. Giricz and co-workers (2003) found an increase in O_2^- production in cholesterol-fed rats, which may explain the decreased cardiac NO-concentration in hyperlipidaemia.

Despite the SFA content of RPO, several clinical trials have evaluated the effect of RPO on blood lipids and lipoproteins. These studies suggest that RPO in a moderate fat, moderate cholesterol diet, despite its high content of SFA, does not raise serum total cholesterol (TC) or LDL cholesterol concentrations (Chandrasekharan, 1999; Theriault *et al.*, 1999; Kritchevsky, 2000; Sundram and Basiron). However, when hypercholesterolaemic subjects and high fat liquid formula diets were used, RPO-supplementation appeared to raise TC and LDL cholesterol (Sundram and Basiron). Little knowledge exists on the protective effects of RPO-supplementation against ischaemia/reperfusion injury, especially with a high-cholesterol diet (Esterhuyse *et al.*, 2003).

Serbinova and co-workers (1992) focused on the functional effects of palm oil vitamin E in the Langendorff perfused rat heart subjected to global ischaemia, while the current study investigated a possible role for the cellular signalling pathways. We therefore designed a study to investigate the effect of RPO on ischaemia/reperfusion injury in the working rat heart model. We also added cholesterol to the diet to simulate an unhealthy dietary-cholesterol intake.

Our main aims were to determine whether: 1) dietary RPO-supplementation of rats on a hypercholesterolaemic diet protected against ischaemia/reperfusion injury, 2) the NO-cGMP pathway can be considered as a possible mechanism of protection and 3) changes in the composition of myocardial total phospholipid fatty acids during ischaemia contributed to this protection.

5.3 Materials and Methods

5.3.1 Experimental Model

Wistar rats were fed a standard rat chow (SRC), or a SRC plus cholesterol diet. In two additional groups, these diets were supplemented with RPO for 6 weeks. The working heart perfusion method used in this Experimental Model is described in Chapter 3 under Materials and Methods (Experimental Model 2 p.58) and the protocol that was followed is summarized in Figure 3.1 p.53.

5.3.2 Measurement of cardiac function

Left ventricular systolic-(LVSP) and diastolic pressure (LVDP), coronary flow (CF), heart rate (HR) and aortic output (AO) were measured as described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50). Functional recovery was expressed as the percentage rate pressure product (RPP) recovery as described in Chapter 3 under Materials and Methods (Experimental Model 2 p.58).

5.3.3 Biochemical analyses

The cAMP and cGMP concentrations were determined using radioimmunoassay kits obtained from Amersham Corp. (Amersham, UK). The principle and methods were discussed in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

5.3.4 Heart muscle total phospholipid fatty acid composition (%)

Freeze-dried myocardial tissue from all the groups was used to determine total phospholipid fatty acid composition by gas chromatography as described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

5.3.5 Serum lipids

Blood was collected from the tail vein of the rats on the SRC diet and groups supplemented with cholesterol and RPO before and after the 6-week supplementation period (n=10 per group). The serum lipid profile was determined using a Beckman Synchron Cx 9-instrument and Beckman Synchron Cx reagents (Beckman Coulter) as described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

5.3.6 Statistical methods

Statistical methods used were described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

5.4 Results

5.4.1 % Rate pressure product recovery

Rate pressure product (RPP) recovery was increased in the RPO-supplemented group when compared with the control group ($89,4 \pm 4,7\%$ *versus* $75,0 \pm 4,7\%$, $P<0,05$) 25 minutes into reperfusion (n=5 per group) (Figure 5.1). RPO also improved RPP recovery in the presence of cholesterol (cholesterol/RPO: $99,9 \pm 4,0\%$ *versus* cholesterol only: $72,1 \pm 2,7\%$, $P<0,05$).

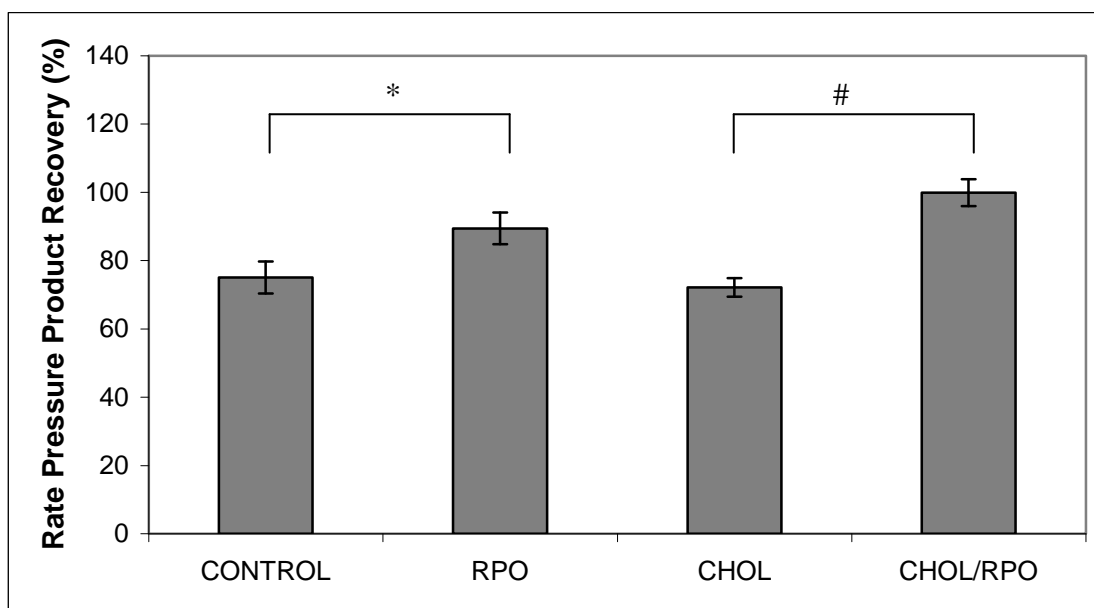


Figure 5.1 % Rate pressure product recovery in the 4 experimental groups 25 minutes into reperfusion (n=5 per group) (* $P<0,05$ for the RPO-supplemented group versus the control group) (# $P<0,05$ for the cholesterol/RPO-supplemented group versus the cholesterol group) (mean \pm SEM)

5.4.2 Aortic output recovery (%)

Results are presented in Figure 5.2. The percentage aortic output recovery of hearts from RPO-supplemented rats was significantly higher than the percentage aortic output recovery in the control hearts, indicating that RPO offered protection against ischaemia/reperfusion injury ($72,9 \pm 3,4\%$ versus $55,4 \pm 2,5\%$, $P<0,05$). Cholesterol-supplementation caused a poor aortic output recovery when compared with the control group ($35,5 \pm 6,2\%$ versus $55,4 \pm 2,5\%$ $P<0,05$, respectively). However, when RPO was added to the cholesterol diet, the percentage aortic output recovery in the cholesterol/RPO-supplemented group was significantly increased when compared with the cholesterol group (cholesterol/RPO: $63,2 \pm 3,1\%$ versus cholesterol: $35,5 \pm 6,2\%$, $P<0,05$).

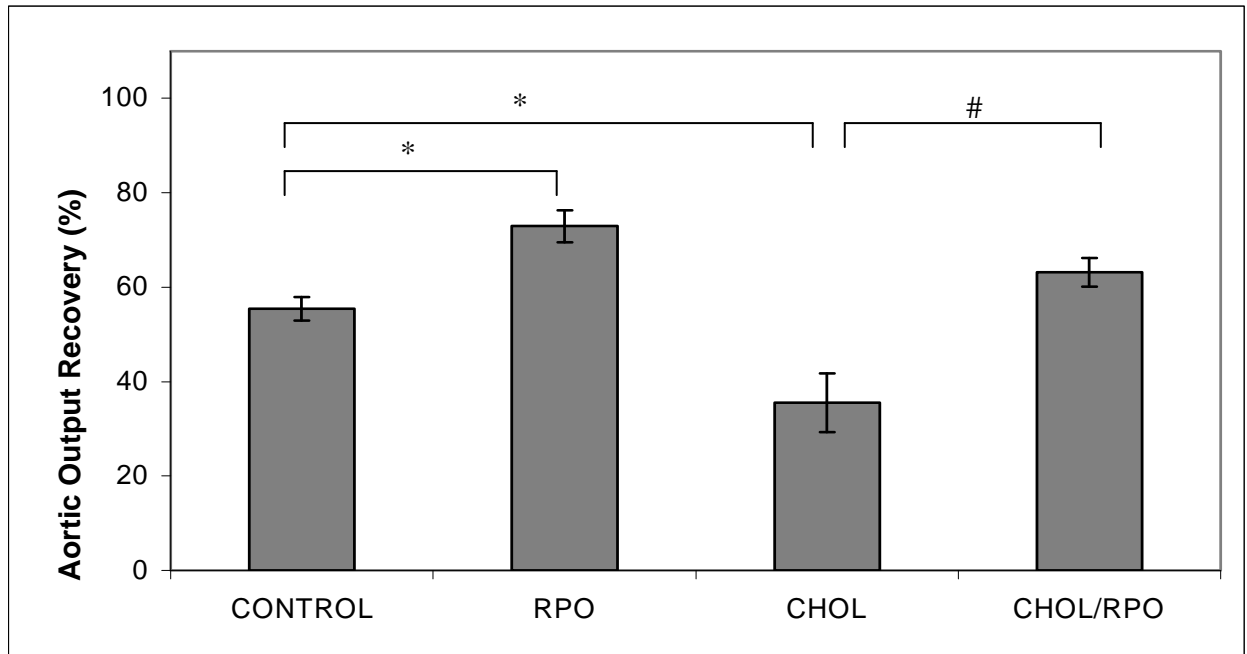


Figure 5.2 % Aortic output recovery in the 4 experimental groups 25 minutes into reperfusion (n=5 per group) (* $P < 0,05$ for the cholesterol- and RPO-supplemented groups *versus* the control group) (# $P < 0,05$ for the cholesterol/RPO-supplemented group *versus* the cholesterol group) (mean \pm SEM)

5.4.3 Effects of RPO-supplementation on ischaemic cAMP and cGMP concentrarions

The cAMP results are presented in Figure 5.3 and cGMP results in Figure 5.4. Baseline values for cAMP and cGMP refer to values at 25 minutes into perfusion before ischaemia was introduced (cAMP: control = $119,9 \pm 7,7$ pmol/g; RPO = $91,7 \pm 6,9$ pmol/g; cholesterol = $97,5 \pm 2,8$ pmol/g; cholesterol/RPO = $112,1 \pm 18,2$ pmol/g) and (cGMP: control = $21,6 \pm 2,4$ pmol/g; RPO = $25,4 \pm 4,0$ pmol/g; cholesterol = $26,1 \pm 2,8$ pmol/g; cholesterol/RPO = $36,4 \pm 9,1$ pmol/g). All values in Figures 5.3 & 5.4 represent the percentage change in cAMP and cGMP concentrations between the baseline value and the value of the corresponding experimental group at 10 and 25 minutes ischaemia and 10 minutes into reperfusion (n=5 per group per time point).

There was a significant difference in myocardial cAMP concentrations between the control group and the RPO-supplemented group at all three sampling points (percent change from baseline: control $19,9 \pm 7,7\%$ *versus* RPO $-8,3 \pm 6,9\%$ at 10 minutes ischaemia; control $6,9 \pm 7,0\%$ *versus* RPO $-11,9 \pm 1,8\%$ at 25 minutes ischaemia and control $30,3 \pm 7,1\%$, *versus* RPO $-3,0 \pm 9,7\%$ at 10 minutes reperfusion) ($P < 0,05$).

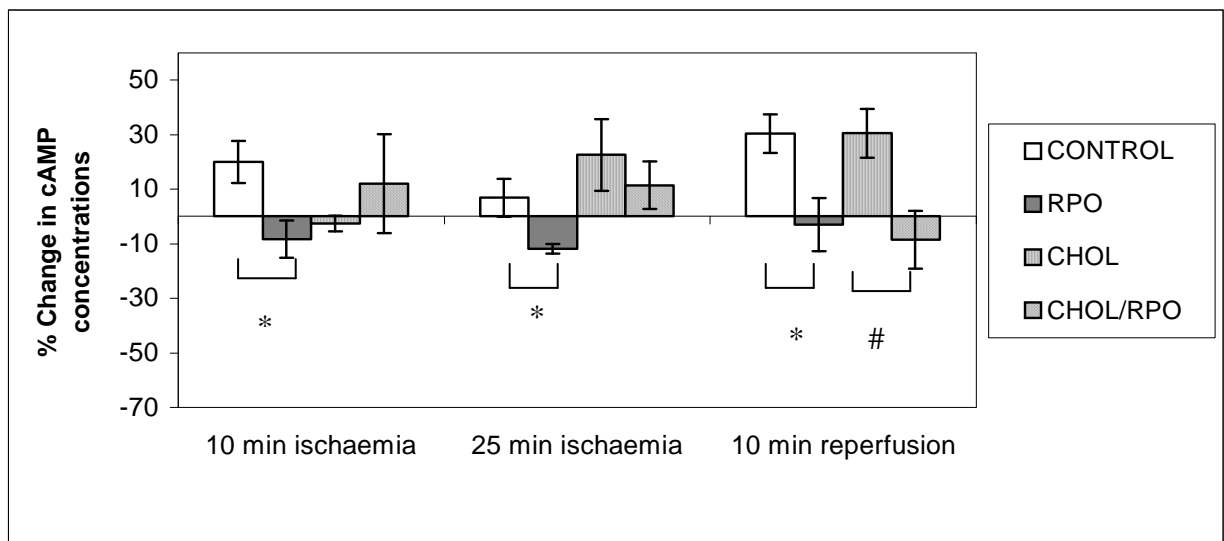


Figure 5.3 % Change in myocardial cAMP concentrations in the 4 experimental groups before ischaemia, during ischaemia and in reperfusion (n=5 per group per time point) (* $P < 0,05$ for the indicated group *versus* the control group) (# $P < 0,05$ for the indicated group *versus* the cholesterol group) (mean \pm SEM)

The cholesterol/RPO-supplemented group showed a significant difference in cAMP at 10 minutes into reperfusion, when compared with the cholesterol group (percent change from baseline: cholesterol/RPO $-8,5 \pm 10,7\%$ *versus* cholesterol $30,6 \pm 8,9\%$,) ($P < 0,05$).

Myocardial cGMP concentrations (Figure 5.4) were significantly increased in the RPO-supplemented group when compared with the control group 10 minutes into ischaemia (RPO $132,9 \pm 36,3\%$ *versus* control $42,7 \pm 24,4\%$) ($P < 0,05$).

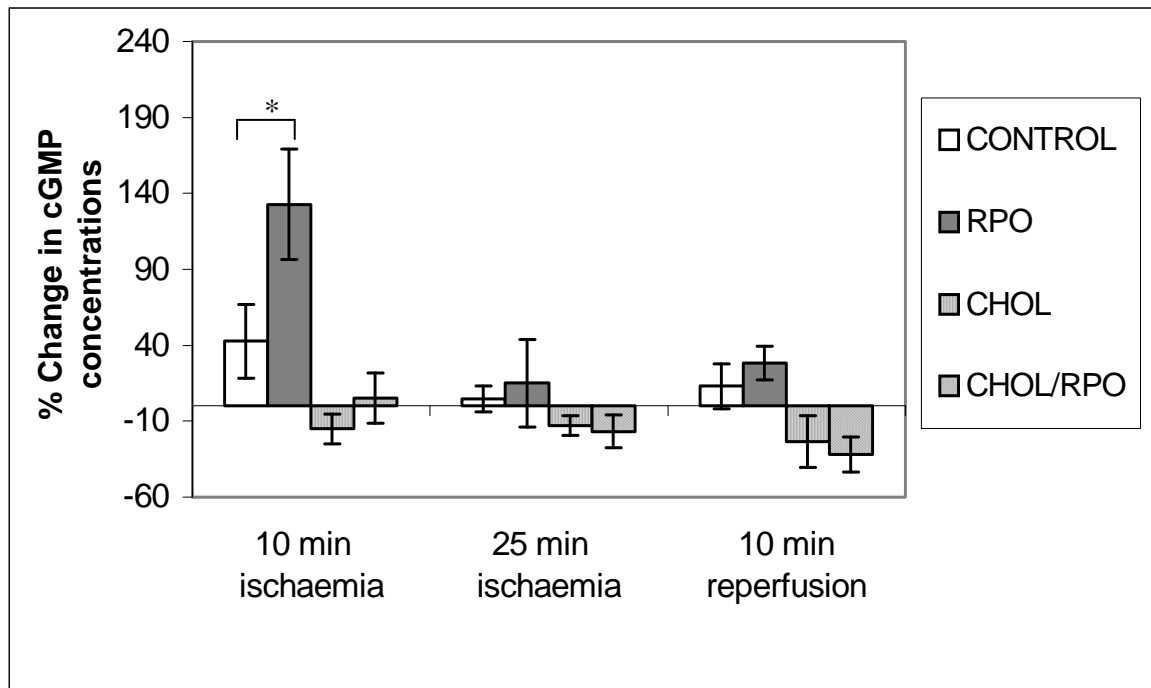


Figure 5.4 % Change in myocardial cGMP concentrations in the 4 experimental groups before ischaemia, during ischaemia and in reperfusion (n=5 per group per time point) (* $P<0,05$ for RPO-supplemented group *versus* the control group) (mean \pm SEM)

5.4.4 Serum lipids

Weight increase of rats remained stable between the control- and supplemented groups after the 6-week supplementation period.

Figure 5.5 - Figure 5.7 represent the percentage change in serum total cholesterol-, high density lipoprotein cholesterol and triacylglycerol concentrations between the baseline value and the value of the corresponding experimental group after a 6-week supplementation period (n=10 per group).

Although there were baseline differences between the control and RPO-supplemented group ($1,3 \pm 0,04$ mmol/l *versus* $1,4 \pm 0,04$ mmol/l, $P<0,05$), the

% change in serum total cholesterol between 4 experimental groups was not different after a 6-week supplementation period (n=10 per group) (Figure 5.5).

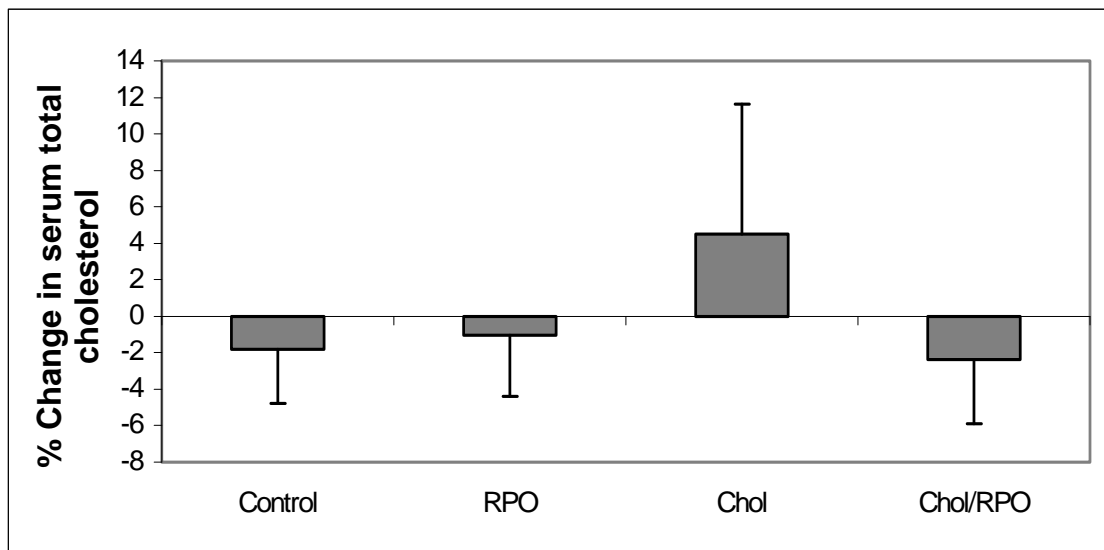


Figure 5.5 % Change in serum total cholesterol in the 4 experimental groups after a 6-week supplementation period (n=10 per group)(mean ± SEM)

HDL-cholesterol followed a similar trend with baseline differences between control and RPO-supplemented groups, but with no difference (% change in serum high density lipoprotein cholesterol) between the experimental groups after the 6-week diet (n=10 per group) (Figure 5.6).

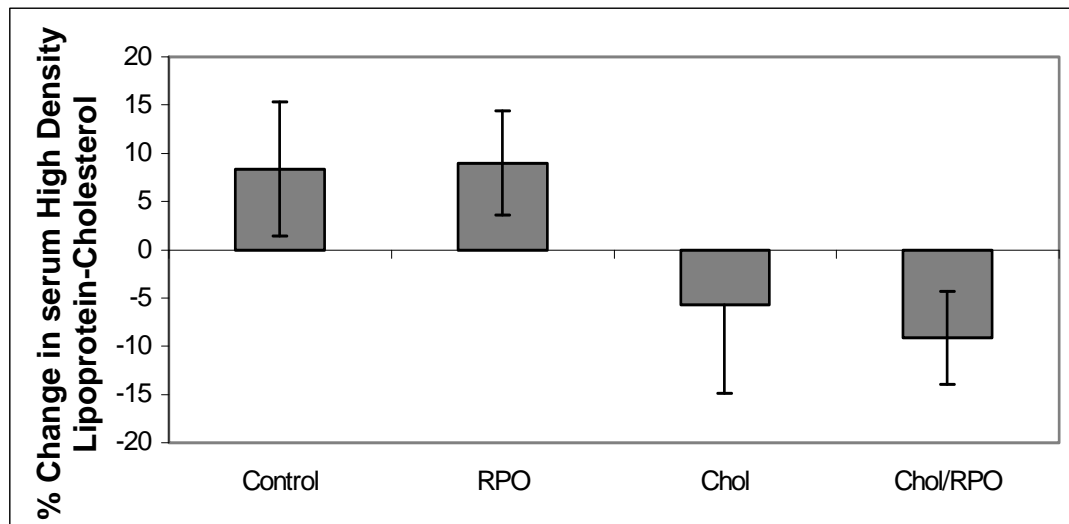


Figure 5.6 % Change in serum high density lipoprotein cholesterol in the 4 experimental groups after a 6-week supplementation period (n=10 per group) (mean ± SEM)

Serum concentrations of triacylglycerol were increased, $P < 0,05$, in the RPO- and cholesterol supplemented groups after 6 weeks on the diet ($0,6 \pm 0,1$ mmol/l and $0,5 \pm 0,1$ mmol/l, respectively before 6-week diet and $0,9 \pm 0,1$ mmol/l and $1,1 \pm 0,2$ mmol/l, respectively after 6-week diet). In the control group it was $0,5 \pm 0,1$ mmol/l before and $0,8 \pm 0,1$ mmol/l after the 6-week diet (n=10 per group) and in the cholesterol/RPO group (n=10) it was $0,8 \pm 0,1$ mmol/l before and $0,9 \pm 0,2$ mmol/l after the 6-week diet. However, the % change in serum triacylglycerols between 4 experimental groups was not different after a 6-week supplementation period (Figure 5.7).

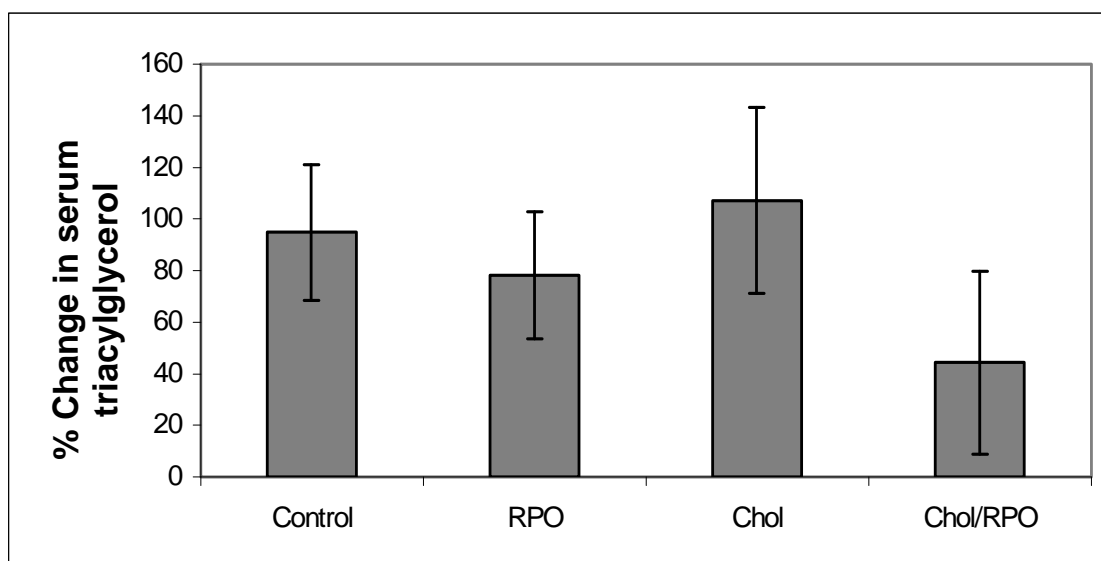


Figure 5.7 % Change in serum triacylglycerols in the 4 experimental groups after a 6-week supplementation period (n=10 per group) (mean ± SEM)

5.4.5 Heart muscle total phospholipid fatty acid composition before and after ischaemia

The major fatty acids of myocardial tissue total phospholipids that play a role in eicosanoid production are presented in Table 5.1. Our results show that dietary supplementation with cholesterol, RPO and cholesterol/RPO caused significant changes in fatty acid composition of myocardial tissue measured before and after ischaemia. The presence of n-3 fatty acids in the standard rat diet were most probably the reason for the high percentage n-3 PUFAs in the myocardial total phospholipids of all the groups. The high percentage myocardial tissue docosahexaenoic acid (DHA) in all the experimental rats may be due to direct incorporation of DHA from the diet, and metabolism of C18:3n-3 and EPA. Results from Table 5.1 show that for DHA, RPO (after ischaemia 17,3%), cholesterol (before ischaemia 19,9%), cholesterol (after ischaemia 19,5%), cholesterol/RPO (before ischaemia 18,8%) and cholesterol/RPO (after ischaemia 18,8%) was significantly different from control (15,9% before and

15,6% after ischaemia, respectively) ($P<0,05$). This occurrence was even more striking in the cholesterol and cholesterol/RPO groups where the high-cholesterol diet may have influenced ALA and EPA metabolism to DHA. The high SFA intake associated with RPO-supplementation could explain the increased myocardial SFAs in RPO- and cholesterol/RPO-supplemented group when compared with the control and cholesterol group before ischaemia, respectively (Table 5.1). Only the RPO-fed rats demonstrated a significant increase in percentage EPA after ischaemia, which may suggest that EPA together with other PUFAs displaced the excess SFAs during ischaemia, which resulted in increased levels of EPA. The percentage MUFAs in the cholesterol group decreased by 0,3% during ischaemia while MUFAs in the control group increased by 0,3% during ischaemia. Cholesterol-supplementation resulted in a lower percentage C18:2n-6 in total phospholipid (TPL) fatty acids compared to the control diet (Table 5.1).

Table 5.1 Major fatty acids (%) of myocardial total phospholipid before and after ischaemia.

	CONTROL		RPO		CHOL		CHOL/RPO	
	Before	After	Before	After	Before	After	Before	After
Total SFA	35.4 ± 0.4	36.1 ± 0.3	38.0 ± 1.3 ^a	33.6 ± 0.2 ^c	35.5 ± 0.3	35.5 ± 0.5	37.5 ± 0.1 ^b	36.4 ± 2.0
Total MUFA	7.5 ± 0.3	7.8 ± 0.8	7.6 ± 0.2	7.4 ± 0.3	6.7 ± 0.2 ^a	6.4 ± 0.2 ^a	6.7 ± 0.2	7.1 ± 0.2
Total PUFA	57.0 ± 1.4	56.0 ± 1.1	54.5 ± 0.5	59.0 ± 0.3 ^c	57.8 ± 0.4	58.0 ± 0.6	55.8 ± 0.3 ^b	56.5 ± 2.0
Total N6	38.8 ± 0.7	38.3 ± 1.3	36.6 ± 1.0	39.0 ± 0.2	35.5 ± 0.6 ^a	35.7 ± 0.6	34.1 ± 0.5	35.2 ± 1.1
Total N3	18.3 ± 0.8	17.7 ± 0.7	17.8 ± 1.3	20.0 ± 0.2	22.4 ± 0.2 ^a	22.3 ± 0.3 ^a	21.7 ± 0.4	21.4 ± 1.2
n-6/n-3 ratio	2.2 ± 0.1	2.2 ± 0.1	2.1 ± 0.2	2.0 ± 0.03	1.6 ± 0.04 ^a	1.6 ± 0.04 ^a	1.6 ± 0.1	1.7 ± 0.1
18:2n-6	22.1 ± 0.6	22.6 ± 1.5	21.5 ± 0.6	22.4 ± 0.3	17.6 ± 1.0 ^a	18.5 ± 0.7 ^a	16.9 ± 0.7	19.1 ± 1.4
20:4n-6	15.5 ± 0.4	14.5 ± 0.4	13.9 ± 0.9	15.4 ± 0.2	16.8 ± 0.4 ^a	16.2 ± 0.6 ^a	16.3 ± 0.4	15.1 ± 0.8
20:5n-3	0.7 ± 0.1	0.6 ± 0.02	0.6 ± 0.1	0.9 ± 0.04 ^{ac}	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.03	0.6 ± 0.1
22:6n-3	15.9 ± 0.7	15.6 ± 0.7	16.0 ± 1.0	17.3 ± 0.2 ^a	19.9 ± 0.2 ^a	19.5 ± 0.3 ^a	18.8 ± 0.5 ^a	18.8 ± 0.7 ^a

SFA=Saturated fatty acids
PUFA=Polyunsaturated fatty acids

MUFA=Monounsaturated fatty acids
TN6= (n-6) Polyunsaturated fatty acids
TN3= (n-3) Polyunsaturated fatty acids

(^ap<0,05 for the group *versus* the Control group)

(^bp<0,05 for the group *versus* the Cholesterol group)

(^cp<0,05 for the group before ischaemia *versus* the corresponding group after ischaemia)

All values in Figure 5.8 represent the percentage change in phospholipid fatty acid composition between the baseline value and the value of the corresponding experimental group at 10 minutes into reperfusion) (n=5 per group per time point). Baseline values for heart muscle total phospholipid fatty acid composition refer to values at 25 minutes into perfusion before ischaemia was introduced (Table 5.1). RPO and cholesterol/RPO-supplemented groups showed a significant decrease in % change in total SFAs when compared with control and cholesterol groups, respectively. Concurrently, the % change in EPAs and total PUFAs increased significantly in hearts of the RPO-

supplemented group when compared with hearts of the control group. Hearts of the cholesterol-supplemented group showed a significant increase in % change of total SFAs when compared with hearts of the RPO-supplemented group, which was associated with a decrease in % change in EPA and total PUFAs in hearts of cholesterol-supplemented group when compared with hearts of RPO-supplemented group.

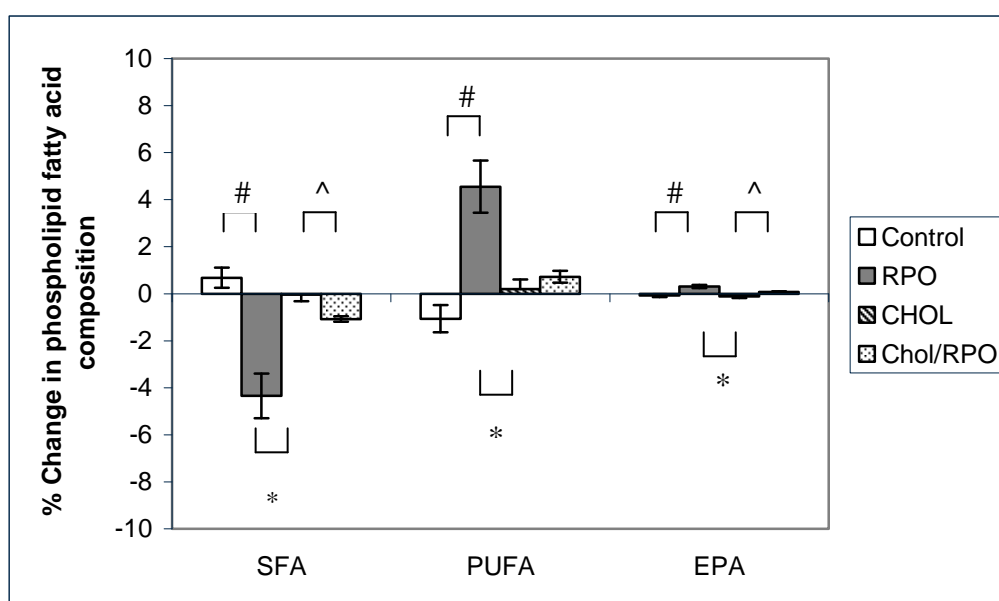


Figure 5.8 % Change in phospholipid fatty acid composition in the 4 experimental groups after an ischaemic period of 25 minutes (n=5 for each group) (# $P<0,05$ for the indicated group versus the control group)(* $P<0,05$ for the group versus the RPO group)(^ $P<0,05$ for the indicated group versus the cholesterol group) (mean \pm SEM)

5.5 Discussion

Diets high in fat and cholesterol are associated with hypercholesterolaemia and are considered a major risk factor for the development of ischaemic heart disease (Ferdinandy *et al.*, 1998; Giricz *et al.*, 2003). To our knowledge little data exist on the effects of dietary RPO-supplementation on post-ischaemic cardiac recovery and the role of 1) the NO-cGMP pathway, 2) total heart

muscle phospholipid fatty acid composition and 3) antioxidant status on this recovery.

The protective properties of NO-cGMP pathway activation could be responsible for improved aortic output recovery of hearts of the RPO-supplemented group when compared with the control-fed animals. This improved functional recovery was associated with elevated cGMP and decreased cAMP concentrations early in ischaemia. However, although our data indicate that RPO-supplementation of a high-cholesterol diet improved functional recovery of the reperfused heart, this improvement could not be linked with altered NO-cGMP signalling. Based on our results we propose that the protective effect of RPO in high-cholesterol diets may be associated with either RPO antioxidant characteristics and/or changes in the phospholipid fatty acid composition of the myocardium during ischaemia/reperfusion. Abeywardena and co-workers (1995) provided supportive evidence for the existence of a complex interaction between fatty acids and antioxidants in RPO-induced protection during ischaemia/reperfusion. These data support the findings of Serbinova and co-workers (1992) which showed that palm oil vitamin E mixture containing both α -tocopherol and α -tocotrienol may be more effective in protecting the heart against ischaemia/reperfusion injury in the isolated Langendorff perfused rat heart than α -tocopherol alone. This higher antioxidant activity of the tocotrienols has been attributed to a number of mechanisms including efficient interaction with free radical species, higher recycling efficiency of chromanoxyl radical and uniform distribution in membrane bilayers (Theriault *et al.*, 1999).

We have previously reported that RPO-supplementation increased total myocardial PUFAs over the 25-minute period of ischaemia (Esterhuyse *et al.*, 2003). In the present study we found that the % EPA in phospholipid (n-3 PUFA) increased from $0,6 \pm 0,1\%$ to $0,9 \pm 0,04\%$ over the same period in the RPO-supplemented group. These findings suggest that EPA, together with other PUFAs, displaced the excess SFAs during ischaemia and thus leads to increased % EPA with subsequent production of DHA. The pathway leading to the biosynthesis of DHA from docosapentaenoic acid (C22:5n-3, DPA) has only recently been deciphered in mammals (Pereira *et al.*, 2003). DPA is elongated to tetracosapentaenoic acid (C24:5n-3), which is then desaturated by a $\Delta 6$ desaturase to generate tetracosahexaenoic acid (C24:6n-3), which is then thought to be transported to the peroxisomes where it undergoes β -oxidation to generate DHA (Figure 2.1 p.12). Cholesterol and cholesterol/RPO-supplemented groups showed increased % DHA phospholipid before ischaemia when compared with control group. All the supplemented groups showed a similar DHA increase after ischaemia when compared with the corresponding control group. The relatively high % DHA in hearts in all the groups, including the control group, could be explained by the composition of the standard rat chow which contains a high percentage of n-3 PUFAs. It is well known that when on high cholesterol diets, membrane fatty acid composition shows increases in the longer chain PUFAs (such as DHA) in order to compensate for, and maintain membrane fluidity to some extent as a result of increased cholesterol molecules in the membrane. Van Rooyen and co-workers (1998) reported that the metabolic rate of EPA in the cell membranes of the erythrocyte decreased in animals consuming a typical

Western diet with high cholesterol content, which would lead to higher levels of EPA. Although our results showed % EPA in phospholipid to be unchanged in cholesterol and cholesterol/RPO-supplemented groups, decreased metabolic rate could explain the increased % DHA in hearts of the cholesterol and cholesterol/RPO-supplemented groups when compared with the other groups. Abeywardena and co-workers (1995) reported that EPA is either poorly incorporated in rat myocardium or further elongated and desaturated to yield DHA, which may explain the high % DHA compared to EPA. Research has shown that DHA, EPA and free AA are antiarrhythmic and protect the heart against ischaemia/reperfusion injury (Li *et al.*, 1997; Nair *et al.*, 1997; Kang and Leaf, 2000; Pepe *et al.*, 2002; Jump, 2003). Our results showed improved aortic output recovery in hearts of rats supplemented with RPO *versus* control hearts and hearts of rats fed cholesterol/RPO *versus* cholesterol-supplementation, although DHA levels were increased in the cholesterol-supplemented group as well. These findings suggest that protection may not only be associated with a change in fatty acid composition and eicosanoid production from dietary intake, but that accompanying non-fatty acid constituents may also be important. Furthermore, research has shown that thromboxane A₂ and prostacyclin production are unlikely to be mediated via fatty acids alone, but that endogenous antioxidants may also play an important role (Abeywardena *et al.*, 1991; Abeywardena and Charnock, 1995; Theriault *et al.*, 1999).

Generally, the mechanism of eicosanoid action is to bind to membrane-receptors, leading to generation of second messengers such as cAMP and

Ca²⁺ (Mohan *et al.*, 1995; Li *et al.*, 1997). In the current study myocardial cAMP concentrations were decreased in the RPO-supplemented group, compared to the other groups. Therefore, prevention of fatal cardiac arrhythmias may depend on the net effect of eicosanoids, which is determined by the status of AA metabolism and the contents of n-6 and other fatty acids (Abeywardena *et al.*, 1991; Charnock *et al.*, 1991; Nair *et al.*, 1997; Kang and Leaf, 2000). Both NO and prostaglandins have important independent effects on cardiovascular function, but the interaction between them determines myocardial performance (Mohan *et al.*, 1995). The mechanism of fatty acid protection remains elusive and needs further investigation.

Research has shown that cholesterol-enriched diets impair NO-cGMP signalling in both endothelial and nonendothelial cells. Girics and co-workers (2003) showed that cholesterol diet-induced hyperlipidaemia decreased cardiac NO concentration, but it does not change nitric oxide synthases (NOS) activity. Therefore, the decreased NO-concentrations were not due to impaired NO synthesis, but caused by breakdown of cardiac NO due to increased ROS production in hyperlipidaemic rats (Girics *et al.*, 2003). These authors also found an increased O₂⁻ production in hyperlipidaemic hearts, which support the assumption that elevated ROS production is responsible for decreased NO concentration in hyperlipidaemic myocardium.

We have previously shown that RPO-supplementation increased cGMP concentrations early in ischaemia when compared with the control-fed rats, which may confer some of the cardioprotection to the ischaemia/reperfused heart (Esterhuyse *et al.*, 2003). Although not indicated in Figure 5.4, the

present study has shown that cholesterol-supplementation for 6 weeks influenced the NO-cGMP signalling pathway by decreasing % change in cGMP concentrations in both the cholesterol and cholesterol/RPO-supplemented groups when compared with the RPO-supplemented group; yet it was not different from the control group. These data are in agreement with other studies, which showed that high-cholesterol diet impairs NO-cGMP signalling in both endothelial and non-endothelial cells (Deliconstantinos *et al.*, 1995).

The decreased myocardial cAMP concentrations in the RPO-supplemented group may be related with increased cGMP concentrations. In this regard it is possible that cGMP may attenuate this type of injury by inhibiting the cAMP-induced increase in the slow inward calcium current, thus leading to a decrease in ischaemic cytosolic calcium concentrations (Sumii *et al.*, 1995; Du Toit *et al.*, 2001).

High cholesterol-diets are also known to inhibit the formation of ubiquinone (coenzyme Q10) production. This coenzyme is a key polyprenyl derivative molecule in the mitochondrial electron transport system and as an endogenous antioxidant it protects the ischaemia/reperfused myocardium (Ferdinandy *et al.*, 1998; Wall, 2000; Girics *et al.*, 2003). CoQ10 is found naturally in small amounts in RPO (Nagendran *et al.*, 2000), which may explain improved aortic output recovery in RPO and cholesterol/RPO-supplemented groups when compared with the control and cholesterol fed groups, respectively.

Supplementation with RPO appeared to raise TC and LDL cholesterol when hypercholesterolaemic subjects and high fat liquid formula diets were used (Sundram and Basiron). However, our results have shown that RPO-supplementation in the presence of potentially harmful cholesterol did not lead to a significant difference in serum total cholesterol. We know that tocotrienols in RPO not only suppress cholesterol production in the liver and lower its serum concentration, but also lower the damaging LDL-cholesterol. Tocotrienols prenylated side-chains are thought to induce prenyl pyrophosphate pyrophosphatase that catalyzes the dephosphorylation of farnesyl with a concomitant increase of cellular farnesol. Farnesol downregulates HMG CoA reductase activity by a post-transcriptional process involving protein degradation. This mechanism is different from cholesterol, which exerts a feedback transcriptional effect on HMG CoA reductase activity (Theriault *et al.*, 1999). These data are in agreement with other studies, which showed that not all saturated dietary fats raise total serum cholesterol (Theriault *et al.*, 1999; Kritchevsky, 2000; Sundram and Basiron).

The findings of this study create opportunities for further investigations to elucidate RPO mediated mechanisms involved in cardiac protection.

5.6 Conclusion

Dietary RPO-supplementation offered protection of hearts from rats on a standard rat chow (control) and hypercholesterolaemic diet against ischaemia/reperfusion injury as reflected by improved aortic output recovery and rate pressure product. This was associated with an increase in cGMP early in ischaemia and a decrease in cAMP during ischaemia in the RPO-

supplemented *versus* control group. However, with addition of cholesterol, cGMP concentrations in ischaemia were decreased, but RPO still offered protection. This suggests that cGMP may not be the only mechanism of protection. The improved functional recovery in the cholesterol/RPO-supplemented group was associated with a significant decrease of cAMP concentrations during reperfusion, suggesting that RPO may also protect via the inhibition of the cAMP pathway.

Furthermore, % total PUFAs in myocardial phospholipids were increased by ischaemia in the RPO-supplemented group, but not in the cholesterol/RPO-supplemented group. This suggests that PUFAs may have influenced the tissue concentrations of cGMP and cAMP.

Evidence in this study suggests that both antioxidants and fatty acids play a role in the cardioprotective mechanisms of dietary RPO and our next aim was to search for the cellular mechanisms whereby antioxidants and fatty acids offer this cardioprotection. We therefore aimed to investigate the effects of RPO-supplementation of rats on a standard rat chow and cholesterol-enriched diets on myocardial NO-synthesis and NO-cGMP signalling.

CHAPTER 6

Proposed mechanisms for red palm oil-induced cardioprotection in a hyperlipidaemic perfused rat heart model.

(Submitted 2005)

6.1 Abstract

Introduction: High-cholesterol diets alter function of the myocardial and vascular NO-cGMP signalling pathway. These alterations have been implicated in both ischaemic/reperfusion injury and the development of ischaemic heart disease. We have previously shown that the cardioprotection offered by red palm oil (RPO)-supplementation could be related to NO-cGMP signalling in the control, but not in the hyperlipidaemic group. **Aims:** We investigated the effects of RPO-supplementation of rats on a standard rat chow (control)- and cholesterol-enriched diet on myocardial nitric oxide synthesis, superoxide dismutase- and nitric oxide synthase activity and lipid hydroperoxide production in the rat heart. **Materials and Methods:** Wistar rats were fed a standard rat chow (SRC), or a SRC plus cholesterol diet. In 2 additional groups, these diets were supplemented with RPO for 6 weeks. Post-ischaemic mechanical function, total myocardial nitric oxide concentrations, lipid hydroperoxide production and superoxide dismutase- and nitric oxide synthase activity were determined in isolated working rat hearts subjected to 25 minutes of normothermic total global ischaemia. **Results:** Dietary RPO-supplementation of the standard rat chow (control) diet and the cholesterol-enriched diet improved aortic output recovery ($72,1 \pm 3,2\%$ *versus* $54,0 \pm 3,2\%$ and $64,0 \pm 2,5\%$ *versus* $39,3 \pm 4,9\%$ respectively, $P < 0,05$). The improved aortic output recovery of hearts from rats supplemented with RPO was associated with an elevation in the cGMP concentration early in ischaemia. Baseline myocardial nitric oxide content was significantly decreased in the cholesterol-enriched diet group when compared to the control group ($1,6 \pm 0,4$ mmol/l *versus* $2,8 \pm 0,2$ mmol/l, $P < 0,05$). Direct intracellular nitric oxide

detection in isolated rat cardiomyocytes showed a significant increase in nitric oxide production after 120-minute simulated ischaemia (hypoxia) in both the RPO- and cholesterol/RPO-supplemented group when compared with non-hypoxic control and cholesterol-supplemented groups, respectively ($2,9 \pm 0,1$ arbitrary units and $2,5 \pm 0,1$ arbitrary units *versus* $2,4 \pm 0,1$ arbitrary units and $2,1 \pm 0,1$ arbitrary units, respectively, $P < 0,05$). **Conclusions:** Our data show that dietary RPO-supplementation protects the hearts of animals on a standard rat chow or hypercholesterolaemic diet against ischaemia/reperfusion injury. Mechanisms for this cardioprotection may include RPO-induced preservation of myocardial ischaemic NO-concentrations, which causes elevated cGMP concentrations in hearts of rats on a standard rat chow diet.

Key words: Hyperlipidaemia - Working heart perfusion - Ischaemia - RPO protection - Nitric Oxide - Free radicals

6.2 Introduction

Nitric oxide (NO) is, by virtue of its vasodilator, antioxidant, anti-platelet and anti-neutrophil actions, an essential molecule for normal heart function (reviewed by Ferdinandy and Schultz, 2003). It has also been shown to be cardioprotective in the ischaemic heart (reviewed by Bolli, 2002). However, NO is detrimental when it is combined with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), which rapidly decomposes to highly reactive oxidant species leading to tissue injury (Figure 2.4). There is a critical balance between cellular concentrations of NO, O_2^- and superoxide dismutase (SOD) which physiologically favour NO production, but in pathological conditions such as ischaemia and reperfusion, result in $ONOO^-$ formation (reviewed by

Ferdinandy and Schultz, 2003). These oxygen free radicals can react with nucleic acids, proteins and lipids, resulting in damage to the cell membrane or intracellular organelles (Gilham *et al.*, 1997).

Protective effects of NO are also mediated through the production of cGMP. Du Toit and co-workers (2001) found that nitric oxide donor treatment reduces ischaemia/reperfusion injury by increasing cGMP concentrations and suggested that the cAMP-to-cGMP ratio might play an important role in this cardioprotection. Maulik and co-workers (1995) showed that NO plays a significant role in transmembrane signalling in the ischaemic myocardium. This group suggested that NO signalling is switched off due to inactivation of NO by reactive oxygen species and were the first to suggest that reactive oxygen species may alter NO-cGMP signalling.

Cardiac stress adaptation is possibly jeopardized in hyperlipidaemia due to altered NO-cGMP pathway function in vascular and myocardial tissue. Szilvassy and co-workers (2001) found that a cholesterol-enriched diet decreased both vascular NO and cGMP concentrations and increased aortic O_2^- production. Several other studies have also shown that a high-cholesterol diet impairs NO-cGMP signalling in both endothelium (Deliconstantinos *et al.*, 1995) and non-endothelial cells with a significant decrease in cardiac NO-concentrations (Ferdinandy *et al.*, 1997; Szekeres *et al.*, 1997). Girics and co-workers (2003) found an increase in O_2^- production with decreased cardiac NO-concentration in cholesterol-fed rats. Nitric oxide synthase (NOS) activity was unchanged which may suggest that NO synthesis was not impaired and

that increased O_2^- production was responsible for the decreased NO-concentrations in the hyperlipidaemic myocardium. Furthermore, hyperlipidaemia stimulates ONOO⁻ generation in the heart, which leads to myocardial dysfunction (Onody *et al.*, 2003).

Diniz and co-workers (2004) showed that changes in dietary fatty acid composition also affect cardiac oxidative stress. They showed that, despite their beneficial effects on serum lipid levels, diets rich in polyunsaturated fatty acids (PUFAs) were deleterious to the heart by increasing cardiac susceptibility to lipid peroxidation. PUFA-fed rats also showed diminished SOD activities when compared to saturated fatty acid (SFA)-fed rats.

We have previously shown that dietary RPO-supplementation protects against the harmful consequences of global ischaemia in the isolated perfused rat heart on a standard rat chow or cholesterol-enriched diet (Esterhuyse *et al.*, 2005). RPO contains a mixture of SFAs (51%), MUFAs (38%), PUFAs (11%) and natural antioxidants, tocopherols and tocotrienols (Nagendran *et al.*, 2000). These natural antioxidants can act as scavengers of damaging oxygen free radicals (Cottrell, 1991; Serbinova *et al.*, 1991; Chandrasekharan, 1999; Theriault *et al.*, 1999; Kritchevsky, 2000). Newaz and co-workers (2003) showed an antioxidant protection by γ -tocotrienols in hypertensive rats when compared with control animals. This group suggested that improved NOS activity in blood vessels and increased NO availability were mediated through the antioxidant properties of γ -tocotrienol where it effectively scavenges the free radicals. Venditti and co-workers (1999) also reported that vitamin E

treatment offers protection against ischaemia/reperfusion-induced oxidative stress. However, the precise mechanism of action remains unknown.

Little is known about the effects and possible protective mechanisms of RPO-supplementation against ischaemia/reperfusion injury, particularly when used as a supplement with a high-cholesterol diet.

The aims of this study were to determine whether RPO-supplementation influences 1) myocardial susceptibility to ischaemia/reperfusion injury in hearts from rats on a standard rat chow or hypercholesterolaemic diet, 2) myocardial NO-cGMP signalling pathway function, 3) production of cardiac NO and lipid hydroperoxides (LPO) and 4) enzymatic NOS and SOD activities.

6.3 Materials and methods

6.3.1 Experimental groups and model used

Wistar rats were fed a standard rat chow (SRC), or a SRC plus cholesterol diet. In 2 additional groups, these diets were supplemented with RPO for 6 weeks. The protocol used has been discussed in Chapter 3 under Materials and Methods (Experimental Model 3 p.60).

6.3.2 Functional parameters measured

Described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

6.3.3 Biochemical parameters measured

6.3.3.1 Measurement of cGMP

Myocardial cGMP concentrations were determined using a radioimmunoassay method as described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

6.3.3.2 Measurement of cardiac nitric oxide concentrations

Myocardial NO concentrations were determined using a Nitrate/Nitrite kit (Cayman Chemicals, Cayman Islands) as described in Chapter 3 under Materials and Methods (Experimental Model 3 p.60). We also measured intracellular myocyte NO-production with DAF-2/DA by flow cytometry (Strijdom *et al.*, 2004) as described in Chapter 3 under Materials and Methods (Experimental Model 3 p.60).

6.3.3.3 Measurement of cardiac nitric oxide synthase activity

Total nitric oxide (NOS) activity was measured using a NOS assay-kit which is based on biochemical conversion of L-arginine to L-citrulline by NOS (Cayman Chemicals, Cayman Islands). For detail about principle and method used, see full description in Chapter 3 under Materials and Methods (Experimental Model 3 p.60).

6.3.3.4 Measurement of cardiac superoxide dismutase activity

Total activity of superoxide dismutase (SOD) was measured as described in Chapter 3 under Materials and Methods (Experimental Model 3 p.60) using a SOD kit (Cayman Chemicals, Cayman Islands).

6.3.3.5 Measurement of cardiac lipid hydroperoxide production

Lipid hydroperoxide (LPO) production in cardiac muscle was assessed using a LPO assay kit (Cayman Chemicals, Cayman Islands) as described in Chapter 3 under Methods and Materials (Experimental Model 3 p.60).

6.3.3.6 Statistical methods

Statistical methods used in this experiment have been discussed in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

6.4 Results

6.4.1 Cardiac functional parameters in isolated perfused rat hearts

In order to compare the functional recovery of the hearts in the different groups, the heart rate (beats/min), coronary flow (ml/min), aortic flow (ml/min) and left ventricular developed pressure (mmHg) were monitored at 20 minutes perfusion and 25 minutes during reperfusion as shown in Table 6.1. Results showed no significant difference between the groups for all parameters measured before and after ischaemia, which suggests that RPO-, cholesterol and cholesterol/RPO-supplementation did not have a negative effect on cardiac function.

Table 6. 1 Cardiac functional parameters in the 4 experimental groups before and after ischaemia

Time Point	Group	HR	CF	AO	LVDevP
20 min Perfusion	Control	271.7± 10.4	21.6±1.9	48.1±2.8	123.4±3.5
	RPO	278.1±19.2	20.4±1.4	46.4±3.2	131.4±4.0
	Chol	237.4± 25.4	21.0±1.7	51.3±3.7	124.1±6.0
	Chol/RPO	202.9±14.1	16.2±1.5	47.2±1.6	124.1±3.7
25 min Reperfusion	Control	244.9±11.8	22.3±1.7	28.6±2.9	101.4±4.1
	RPO	283.1±15.8	21.3±1.9	33.4±2.5	114.4±5.5
	Chol	212.9±12.8	23.0±3.6	23.6±4.1	98.6±6.8
	Chol/RPO	209.0±23.3	17.4±2.0	31.3±1.8	121.0±3.7

Working heart perfusion cardiac functional parameters of control and cholesterol-fed rats with/without red palm oil-supplementation for 6 weeks. Heart rate (HR, beats/min); Coronary flow (CF, ml/min); Aortic output (AO, ml/min); Left ventricular developed pressure (LVDevP). Values are mean ± SEM (n= 7 in each group)

6.4.1.1 Aortic output recovery (%)

Results are presented in Figure 6.1. The percentage aortic output recovery of hearts of the group supplemented with RPO was higher than in the hearts of the control group, suggesting that RPO protected against the consequences of ischaemia/reperfusion ($72,1 \pm 3,2\%$ versus $54,0 \pm 3,2\%$, $P<0,05$) (n=7 per

group per time point). Cholesterol-supplementation caused a poor aortic output recovery when compared with the control group ($42,9 \pm 6,3\%$ versus $54,0 \pm 3,2\%$, $P < 0,05$). However, when RPO was added to the cholesterol diet, the percentage aortic output recovery of hearts from rats in the cholesterol/RPO-supplemented group was significantly increased when compared with aortic output recovery of hearts from the cholesterol-fed group ($64,7 \pm 2,4\%$ versus $42,9 \pm 6,3\%$, $p < 0,05$) ($n = 7$ per group).

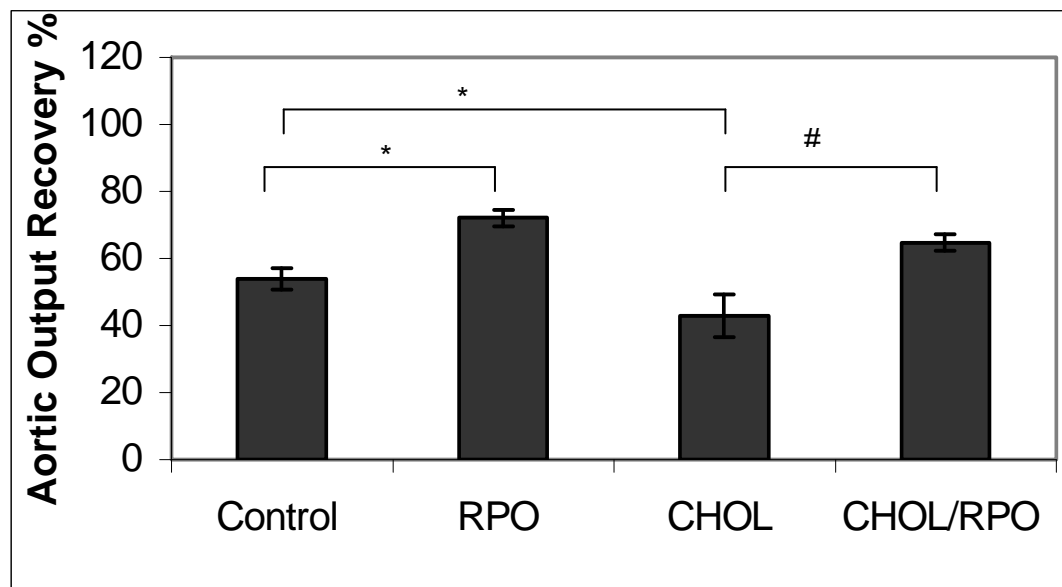


Figure 6.1 % Aortic output recovery of hearts from rats fed a standard rat chow (control) or standard rat chow plus cholesterol and/or RPO-supplementation 25 minutes into reperfusion ($n = 7$ per group) (* $P < 0,05$ for the indicated group versus the control group) (# $P < 0,05$ for the indicated group versus the cholesterol group) (mean ± SEM)

6.4.2 Cardiac cGMP concentrations

Baseline cGMP concentrations refer to values at 25 minutes into perfusion before ischaemia was introduced (control = $21,6 \pm 2,4$ pmol/g; RPO = $25,4 \pm 4,0$ pmol/g; cholesterol = $26,1 \pm 2,8$ pmol/g and cholesterol/RPO = $36,4 \pm$

9,1pmol/g). All values in Figure 6.2 represent the percentage change in cGMP concentrations between the baseline value and the value of the corresponding supplemented group at 10 and 25 minutes ischaemia and 10 minutes into reperfusion. The cGMP concentrations were significantly increased in hearts of the group supplemented with RPO when compared with hearts of the control group 10 minutes into ischaemia (RPO $132,0 \pm 36,3\%$ versus control $42,7 \pm 24,4\%$, $P<0,05$) (n=5 per group per time point).

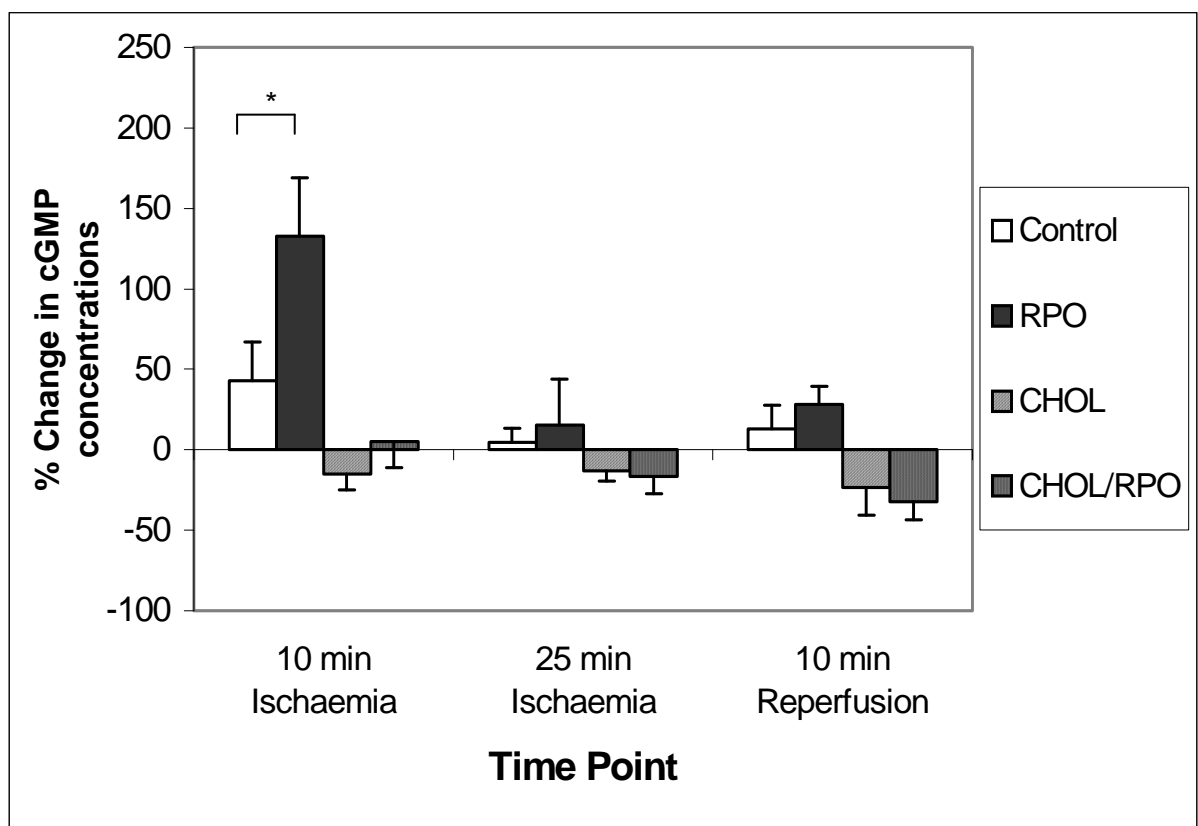


Figure 6.2 % Change in myocardial cGMP concentrations in the 4 experimental groups between the baseline value and the value of the corresponding supplemented group at 10 and 25 minutes ischaemia and 10 minutes into reperfusion. (n=5 per group per time point) (* $P<0,05$ for the indicated group versus the control group) (mean \pm SEM)

6.4.3 Cardiac nitric oxide content

Baseline myocardial NO content was significantly decreased in the cholesterol-enriched diet group when compared to the control group ($1,6 \pm 0,4$ mmol/l *versus* $2,8 \pm 0,2$ mmol/l, $P<0,05$) (Figure 6.3).

However, baseline myocardial NO content of the RPO-supplemented group showed no significant difference when compared with control group. Similarly, the cholesterol/RPO-supplemented group was unchanged when compared with cholesterol-enriched group. Myocardial NO concentrations were also significantly decreased in the cholesterol-enriched diet group when compared to the control group at 10 minutes ischaemia ($2,1 \pm 0,2$ mmol/l *versus* $4,4 \pm 1,2$ mmol/l, $P<0,05$), but similar in all the other groups and also when compared with baseline values (Figure 6.3).

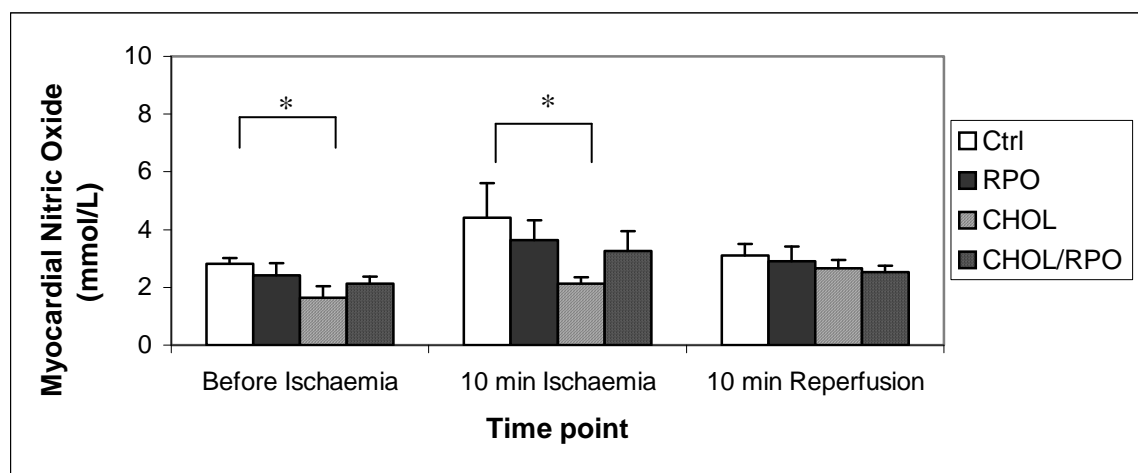


Figure 6.3 Total myocardial NO concentrations in the 4 experimental groups before ischaemia, during ischaemia and in reperfusion (* $P<0,05$ for the indicated group *versus* the control group) (n=5 per group per time point) (mean \pm SEM)

However, direct intracellular NO detection in isolated rat cardiomyocytes showed a significant increase in NO production after 120 minutes of simulated ischaemia (hypoxia) in the RPO-supplemented group when compared with

baseline (non-hypoxic) value in RPO-supplemented group and hypoxic control group ($2,9 \pm 0,1$ arbitrary units *versus* $2,5 \pm 0,1$ arbitrary units and $2,4 \pm 0,1$ arbitrary units, respectively, $P<0,05$) (Fig 6.4A). The cholesterol/RPO-supplemented group showed a similar increase in myocardial NO production

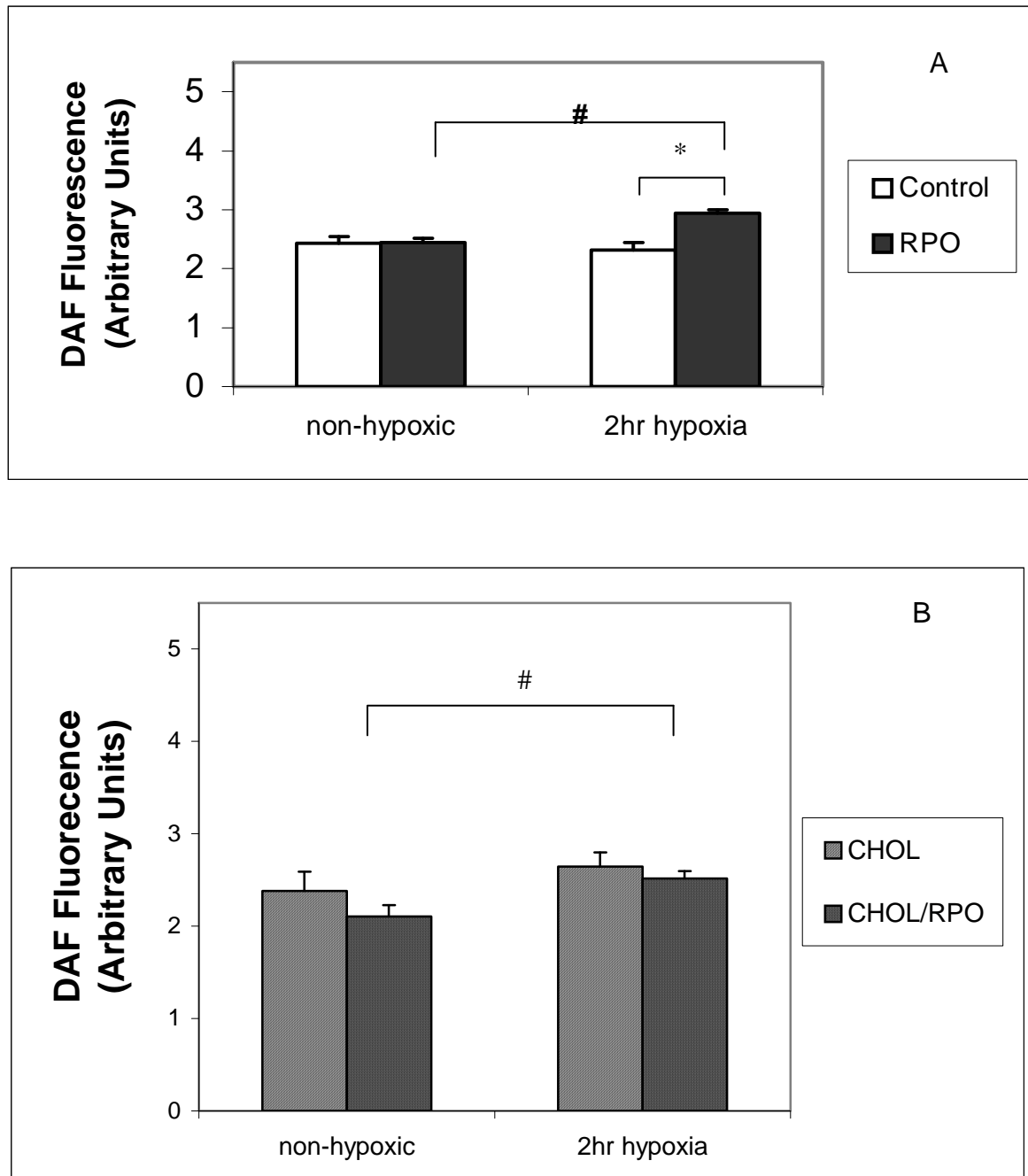


Figure 6.4 Intracellular nitric oxide as detected by DAF fluorescence in isolated cardiomyocytes. (A) (* $P<0,05$ for the indicated group *versus* the control group) (# $P<0,05$ for the hypoxic group *versus* the non-hypoxic group) (n=6) (B) (# $P<0,05$ for the hypoxic group *versus* the non-hypoxic group) (n=6) Experimental groups consist of samples from different hearts (mean \pm SEM)

after 120 minutes of simulated ischaemia (hypoxia) when compared with baseline NO-concentration in cholesterol/RPO-supplemented group ($2,5 \pm 0,1$ arbitrary units *versus* $2,1 \pm 0,1$ arbitrary units, $P<0,05$) (Fig 6.4B).

6.4.4 Cardiac nitric oxide synthase activity

Myocardial NOS activity was significantly decreased in the control group at 10 minutes ischaemia when compared with baseline values in control group ($5,7 \pm 0,3\%$ *versus* $3,9 \pm 0,5\%$, $P<0,05$) ($n=5$ per group per time point) (Figure 6.5).

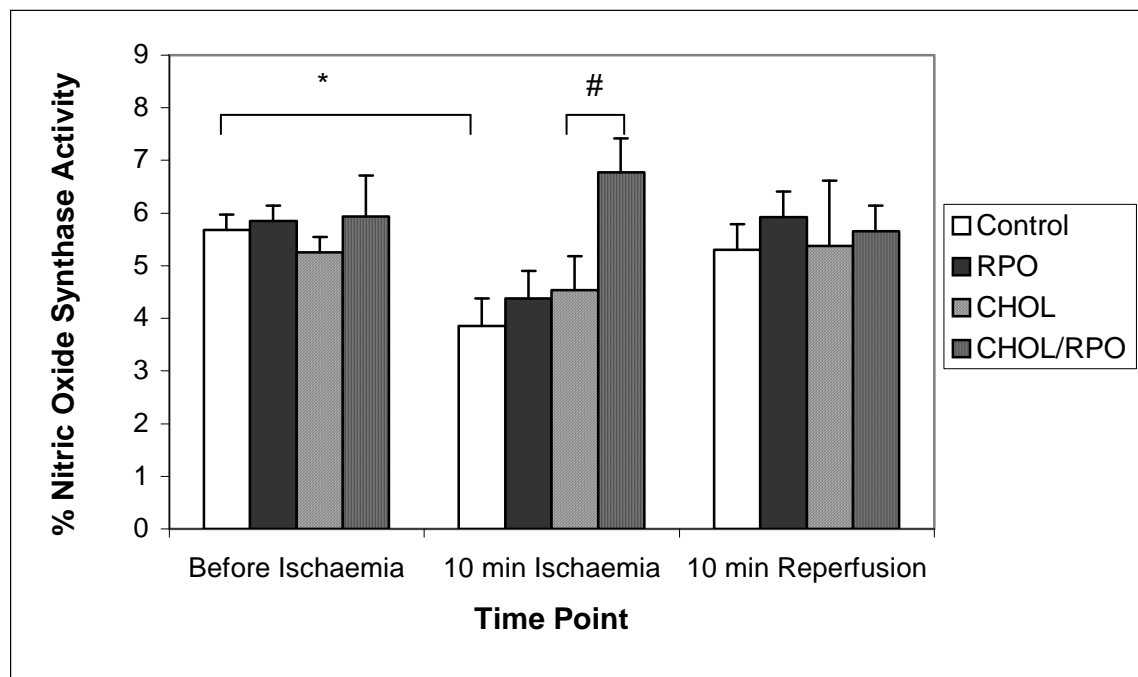


Fig 6.5 % Myocardial NOS activity in the 4 experimental groups before ischaemia, during ischaemia and in reperfusion ($n=5$ per group per time point) (* $P<0,05$ for the indicated group *versus* the same group before ischaemia) (# $P<0,05$ for the indicated group *versus* the cholesterol group) (mean \pm SEM)

Baseline myocardial NOS activity was unchanged for cholesterol-, cholesterol/RPO- and RPO-supplemented groups. However, myocardial NOS activity was significantly increased in cholesterol/RPO-supplemented group

when compared with cholesterol group at 10 minutes ischaemia ($6,8 \pm 0,6\%$ versus $4,5 \pm 0,6\%$, $P<0,05$) (n=5 per group per time point).

6.4.5 Cardiac superoxide dismutase activity

Baseline (20-minute perfusion), ischaemic- and reperfusion SOD activity was similar in all groups at all time points investigated (Table 6.2).

6.4.6 Cardiac lipid hydroperoxide production

Direct measurement of myocardial lipid hydroperoxides in rats fed a standard rat chow or a standard rat chow plus cholesterol and/or RPO-supplementation showed no significant difference in baseline-, ischaemic- and reperfusion hydroperoxide levels when compared with the control group (Table 6.2).

Table 6.2 Baseline (20-minute perfusion), ischaemic and post-ischaemic myocardial super oxide dismutase activity and lipid hydroperoxide levels in the 4 experimental groups (n=5 per group per time point)

	Group	20 minutes perfusion	10 minutes ischaemia	10 minutes reperfusion
SOD (U/ml)	Control	1.6±0.2	1.9±0.5	1.9±0.1
	RPO	1.8±0.4	1.6±0.1	2.1±0.2
	Chol	2.3±0.3	2.0±0.4	1.7±0.3
	Chol/RPO	1.9±0.2	2.0±0.3	2.4±0.1
LPO (nM/L)	Control	10.4±0.4	11.9±0.8	10.1±0.3
	RPO	9.2±0.5	9.5±0.7	9.4±0.2
	Chol	9.5±0.5	9.7±0.7	9.3±1.4
	Chol/RPO	9.6±1.0	9.5±1.2	10.1±1.8

SOD = Superoxide dismutase
LPO = Lipid hydroperoxide
(values are mean ± SEM)

6.5 Discussion

Our data show that RPO-supplementation of a standard rat chow (control) or cholesterol-enriched diet improves aortic output recovery when compared with control and cholesterol-fed groups, respectively. The improved functional recovery in hearts from rats on a control diet supplemented with RPO was associated with an elevation in ischaemic cGMP concentration. Cholesterol-supplementation decreased baseline myocardial NO-concentration when compared with the control group, but the decrease was not associated with changes in cardiac NOS activity. Intracellular NO concentrations in isolated cardiomyocytes from RPO-supplemented rats were increased after 120 minutes of simulated ischaemia (hypoxia) when compared with baseline (non-hypoxic) value in RPO-supplemented group and hypoxic control group. The cholesterol/RPO-supplemented group showed a significant increase in myocardial NO production after hypoxia compared to the non-hypoxic conditions, a trend that was not observed in hearts of cholesterol-fed rats without RPO-supplementation. Ischaemia decreased myocardial NOS activity in the control hearts. However, hearts of the cholesterol/RPO-supplemented group showed a significant increase in ischaemic NOS activity compared to hearts of the cholesterol-supplemented group.

6.5.1 Effects of cholesterol-enriched diet on baseline myocardial NO concentrations

It is well known that hyperlipidemia leads to increased production of ROS in the vasculature which in turn leads to formation of ONOO^- (Deliconstantinos *et al.*, 1995). Onody and co-workers (2003) showed that a cholesterol-enriched

diet reduces cardiac NO concentrations, enhances cardiac formation of O_2^- and stimulates $ONOO^-$ generation in the heart, which leads to myocardial dysfunction. Another study by the same group confirmed these results, but also showed that a cholesterol-enriched diet does not change NOS activity (Girics *et al.*, 2003). They speculated that the decreased myocardial NO concentrations were not due to impaired NO synthesis, but was possibly caused by increased breakdown of cardiac NO due to hyperlipidaemia (Ferdinandy *et al.*, 1998; Girics *et al.*, 2003). The mechanism of decreased myocardial NO level in hyperlipidaemia is still unknown. Girics and co-workers (2003) further propose that increased superoxide production in hyperlipidaemic hearts would promote the reaction of ROS with NO to form $ONOO^-$ and support the assumption that elevated ROS production is responsible for decreased NO concentrations in the hyperlipidaemic myocardium.

6.5.2 NO production during ischaemia

Our results showed that myocardial NOS activity was impaired under ischaemic conditions in control hearts when compared with baseline (non-ischaemic) values of the same group. Surprisingly, ischaemic myocardial NOS activity of hearts from the cholesterol/RPO-supplemented group was increased when compared with cholesterol-supplemented group. These findings are supported by the data obtained with the isolated cardiomyocytes. The hearts of rats supplemented with cholesterol/RPO showed an increased intracellular cardiomyocyte NO production after 120 minutes of simulated ischaemia (hypoxia) when compared with baseline NO-concentration in cholesterol/RPO-supplemented group. Newaz and co-workers (2003) demonstrated antioxidant-

mediated protection by γ -tocotrienols in hypertensive rats. This group suggested that improved NOS activity in blood vessels and increased NO availability were mediated through the antioxidant properties of γ -tocotrienol, which effectively scavenges the free radicals. Previous studies (Onody *et al.*, 2003) have demonstrated that a cholesterol-enriched diet increased basal myocardial superoxide generation. Based on these data we speculate that free radical generation would be further increased during ischaemia. Elevated concentrations of free radicals may contribute to the suppressed NOS activity in the ischaemic hearts of the cholesterol fed rats. The inclusion of RPO containing tocopherols and tocotrienols, could potentially improve NOS activity in the cholesterol/RPO-supplemented group by reducing free-radical induced NOS enzyme damage.

Although there appears to be a discrepancy between ischaemic NOS activity and NO concentrations in the hearts, these data may be explained by the findings of Zweier and co-workers (1999). These authors have shown that NO generation from nitrite via a NOS-independent pathway can be the major source of NO during ischaemia.

6.5.3 Dietary vitamin E and generation of NO, O_2^- and $ONOO^-$ in cholesterol-enriched diets

Although our data indicate that RPO-supplementation of rats on a high-cholesterol diet improved functional recovery of the ischaemic/reperfused heart, this improvement could not be linked with increased NO-cGMP signalling, despite the increased intracellular cardiomyocyte NO concentrations as observed in the cholesterol/RPO-supplemented group after 120-minute

hypoxia when compared with the non-hypoxic cholesterol/RPO-supplemented group. Research has shown that cholesterol-enriched diets impair NO-cGMP signalling in both endothelial and non-endothelial cells (Deliconstantinos *et al.*, 1995; Ferdinandy *et al.*, 1997; Szekeres *et al.*, 1997; Szilvassy *et al.*, 1997). Therefore, based on our results we propose that the protective effect of RPO in high-cholesterol diets may be due to the dietary RPO vitamin E antioxidant characteristics. Vitamin E acts as a free radical scavenger that can react with oxygen, superoxide anion radical and hydroxyl radical (Abudu *et al.*, 2004). Due to its lipid solubility, it is predominantly a chain breaking antioxidant within the lipoprotein (Abudu *et al.*, 2004).

Chow and co-workers (2002) reported that dietary vitamin E is capable of reducing the production and/or availability of not only O_2^- , but also NO and ONOO $^-$. By reducing available O_2^- and NO, vitamin E may alleviate nitric oxide toxicity via reduced formation of reactive ONOO $^-$.

However, it is not clear if the action of vitamin E to reduce the generation of O_2^- and other ROS is independent of its antioxidant function.

6.5.4 RPO-supplementation and NO-cGMP signalling

Improved functional recovery of hearts of rats supplemented with RPO when compared with hearts of rats on a control diet was associated with elevated cGMP concentrations early in ischaemia. Increased intracellular cardiomyocyte NO concentrations as observed in the RPO-supplemented group after 120-minute hypoxia may contribute to the elevated cGMP concentration and may confer some of the cardioprotection to the ischaemic/reperfused heart. Maulik and co-workers (1995) showed that NO plays a significant role in

transmembrane signalling in the ischaemic myocardium. The signalling seems to be transmitted via cGMP and they suggested that NO signalling is switched on and off due to inactivation of NO by reactive oxygen species. Therefore, if dietary RPO containing vitamin E antioxidants act as free radical scavengers, elevated cGMP concentrations in early ischaemia through increased nitric oxide signalling could be responsible for improved functional recovery in hearts of rats supplemented with RPO *versus* non-RPO fed rat hearts in the control group.

6.5.6 Effects of diets rich in PUFAs and SFAs in cardiovascular disease

In a previous study we reported an increase in baseline myocardial total % SFA composition of total phospholipid with RPO-supplementation of the control and cholesterol groups, respectively (Esterhuyse *et al.*, 2005).

Diniz and co-workers (2004) reported that changes in dietary fatty acids affect cardiac oxidative stress. They showed that diets rich in PUFAs, despite the beneficial effects on serum lipids, were deleterious when compared with SFAs in the heart by increasing cardiac susceptibility to lipid peroxidation. Their observation that SFA-fed rats had lower myocardial hydroperoxide concentrations than PUFA-fed rats demonstrates the importance of the PUFA:SFA ratio on lipid peroxidation and the level of antioxidants. They also showed that PUFA-fed rats had diminished SOD activity.

We have also reported in a previous study that RPO-supplementation of rats on a standard rat chow (control) increased total myocardial PUFAs over the

25-minute period of ischaemia (Esterhuyse *et al.*, 2003). Bagchi and co-workers (1998) reported that vitamin E protects PUFAs in cell membranes from peroxidation, which may confer some of the cardioprotection in RPO-supplemented control and cholesterol-treated rats.

The findings of this study create opportunities for further investigations to elucidate RPO mediated mechanisms involved in cardiac protection.

6.6 Conclusion

Our data show that RPO-supplementation improves aortic output recovery in hearts from both standard rat chow (control) and cholesterol fed animals. The improved functional recovery seen in control RPO-supplemented hearts may be due to preservation of ischaemic myocardial NO and cGMP concentrations. However, RPO-supplementation of a high cholesterol diet probably protects the isolated rat heart against ischaemia/reperfusion injury by mechanisms independent of the NO-cGMP signalling pathway.

Myocardial hypoxia-reoxygenation is associated with upregulation of a number of endogenous enzymes, including the matrix metalloproteinases (MMPs), which can induce apoptosis with subsequent exacerbation of cardiac dysfunction (Chen *et al.*, 2003). Therefore, the next study was designed to determine whether RPO-supplementation offers cardioprotection during ischaemia/reperfusion by influencing the regulation of both mitogen-activated protein kinases and serine/threonine protein kinases.

CHAPTER 7

p38-MAPK and PKB/Akt, possible role players in red palm oil-induced protection of the isolated perfused rat heart?

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7.1 ABSTRACT

Introduction: It has been shown that dietary red palm oil (RPO) supplementation improves reperfusion function. However, no exact protective cellular mechanisms have been established. **Aims:** To determine a potential mechanism for functional improvement by investigating the regulation of both mitogen-activated protein kinases (MAPKs) and serine/threonine protein kinases (PKB/Akt) in the presence, and absence, of dietary RPO-supplementation in ischaemia/reperfusion. **Materials and Methods:** Wistar rats were fed a standard rat chow (control) diet or standard rat chow diet plus 7g RPO per kg diet for 6 weeks. Hearts were excised and mounted on an isolated working heart perfusion apparatus. Cardiac function was measured before and after hearts were subjected to 25 minutes of total global ischaemia. Hearts subjected to the same conditions were freeze-clamped and used to characterize the degree of phosphorylation of extracellular signal-regulated kinase (ERK), p38, c-Jun NH₂-terminal protein kinase (JNK) and PKB/Akt. **Results:** Dietary RPO-supplementation significantly improved aortic output recovery ($72.1 \pm 3.2\%$ *versus* $54.0 \pm 3.2\%$, $P < 0.05$). This improved aortic output recovery was associated with significant increases in p38- and PKB/Akt phosphorylation during reperfusion when compared with control hearts. Furthermore, a significant decrease in JNK phosphorylation and attenuation of PARP cleavage occurred in the RPO-supplemented group during reperfusion. **Conclusions:** Our results suggest that dietary RPO-supplementation caused differential phosphorylation of the MAPKs and PKB/Akt during ischaemia/reperfusion-induced injury. These changes in phosphorylation were associated with improved functional recovery and reduced cleavage of an

apoptotic marker, arguing that dietary RPO-supplementation may confer protection via the MAPK and PKB/Akt signalling pathways during ischaemia/reperfusion induced injury.

7.2 Introduction

Cardiovascular disease remains one of the major causes of death in modern society. Although it was previously shown that dietary red palm oil (RPO)-supplementation protects against ischaemia/reperfusion injury in the isolated perfused rat heart (Esterhuyse *et al.*, 2005), the mechanism of action of RPO remains to be elucidated.

Several signal transduction pathways in the heart are regulated in direct response to ischaemia/reperfusion-induced injury. One of the best-characterized signal transduction pathways in the heart is the family of mitogen-activated protein kinases (MAPKs). The MAPKs are a family of serine-threonine kinases that are activated in response to a variety of extracellular stimuli (Robinson and Cobb, 1997; Ip and Davis, 1998). Three major MAPKs including extracellular signal-regulated protein kinase (ERK), p38, and c-Jun NH₂-terminal protein kinase (JNK), have been implicated in the response to ischaemia and reperfusion in the heart (Bogoyevitch *et al.*, 1996; Knight and Buxton, 1996). All three MAPKs have been shown to play pivotal roles in transmission of signals from cell surface receptors to the nucleus and are involved in cell growth, differentiation and apoptosis (Mansour *et al.*, 1994; Leppa *et al.*, 1998; Nemoto *et al.*, 1998). Another potential target of RPO might be the serine/threonine kinase PKB/Akt. PKB/Akt contains a pleckstrin homology (PH) domain that is part of a slightly larger portion in the NH₂

terminus, called the Akt homology domain. The phosphoinositide 3-kinase (PI3-K) product phosphatidylinositol-3,4-bisphosphate binds *in vitro* directly to the PH domain and increases enzyme activity (Downward, 1998). PKB/Akt has been shown to be activated by factors that stimulate PI3-K including thrombin, platelet-derived growth factor and insulin (Downward, 1998). There is also increasing evidence that the PKB/Akt pathway participates in ischaemia/reperfusion-induced injury (Brar *et al.*, 2002; Andreucci *et al.*, 2003).

Very little information regarding the effects of fatty acids and antioxidants (major components of RPO) on the MAPK family and PKB is available in the heart. Chen and co-workers (2003) reported that eicosapentaenoic acid inhibits hypoxia-reoxygenation-induced injury by attenuation of p38 MAPK. Furthermore, it was reported that antioxidant treatment of myocytes suppressed the increase in ROS and blocked ERK activation and the subsequent cardiac hypertrophy induced by these stimuli (Tanaki *et al.*, 2001).

However, as far as we know, no evidence exists for an interaction between RPO and the activation/inhibition of the MAPKs and the pro-survival kinase, PKB during ischaemia and reperfusion. In order to assess the possible mechanisms of protection, the isolated perfused rat heart model was used to determine whether dietary RPO-supplementation was associated with changes in the regulation of the MAPKs and PKB/Akt during ischaemia and reperfusion.

7.3 Materials and Methods

7.3.1 Antibodies and chemicals

Antibodies were purchased from Cell Signalling Technology and all other chemicals were obtained from Sigma (St Louis, MO).

7.3.2 Experimental groups and model used

Wistar rats were fed a standard rat chow (control) diet or control diet plus 7g RPO per kg diet for 6 weeks. The working heart perfusion method used in this experiment, as well as the methods for assessment of myocardial MAPKs activities, have been discussed in Chapter 3 under Materials and Methods (Experimental Model 4 p.65).

7.3.3 Functional parameters measured

Post-ischaemic mechanical function was measured as described in Chapter 3 under Materials and Methods (Experimental Model 1 p.50).

7.3.4 Western blot analysis

Hearts were freeze-dried and used to characterize the degree of activation (i.e. phosphorylation) of extracellular signal-regulated kinase (ERK), p38, c-Jun NH₂-terminal protein kinase (JNK) and PKB/Akt as described in Chapter 3 under Materials and Methods (Experimental Model 4 p.65).

7.3.5 Statistical methods

Statistical methods used in this experiment have been discussed in Chapter 3 under Materials and Methods (Experimental Model 4 p.65).

7.4 Results

7.4.1 Aortic output recovery (%)

We used aortic output recovery as an indirect index of the severity of ischaemia/reperfusion injury. These data suggests that RPO protected against the consequences of ischaemia/reperfusion (RPO $72,1 \pm 3,2\%$ *versus* control $54,0 \pm 3,2\%$, $P<0.05$) (n=7 per group) (Figure 7.1).

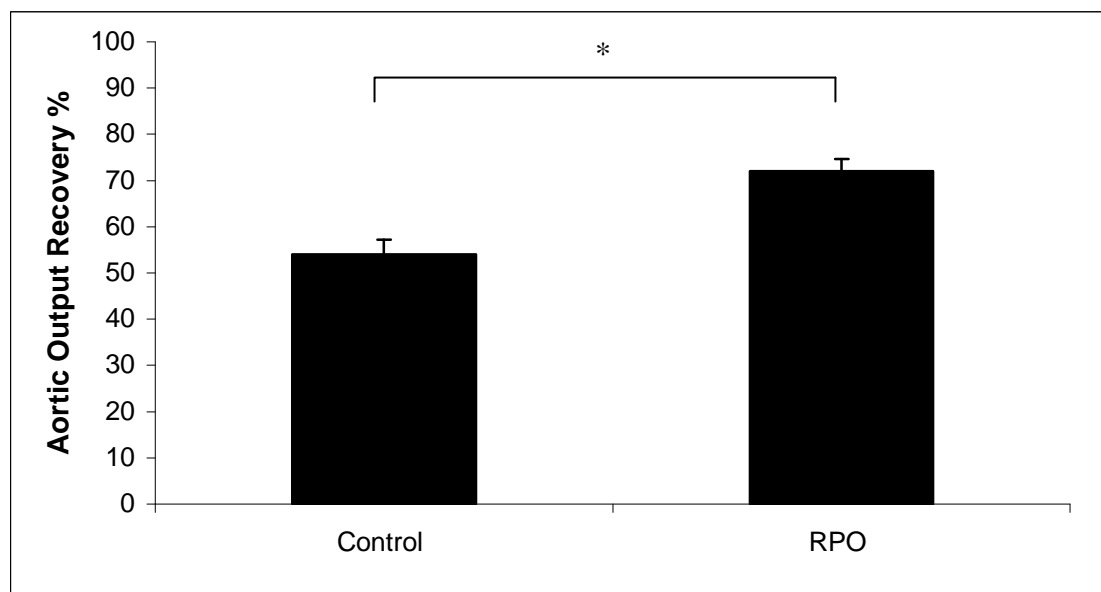


Figure 7.1 % Aortic output recovery of hearts from RPO-supplemented group *versus* control group. (n=7 per group) (* $P<0,05$ for RPO-supplemented group *versus* control group) (mean \pm SEM)

7.4.2 The effect of RPO-supplementation on the phosphorylation of p38, JNK and ERK in hearts subjected to ischaemia and reperfusion

Phosphorylation of p38, JNK (p46/p54-MAPK) and ERK 1/2 (p42/p44-MAPK) was determined by Western blotting using phospho-specific antibodies. As

shown in Figure 7.2A, p38 phosphorylation was significantly increased in the RPO-supplemented group during reperfusion *versus* the control (reperfusion) group (RPO: 4.42 ± 0.35 fold *versus* control: 1.84 ± 0.39 fold, $P < 0.001$).

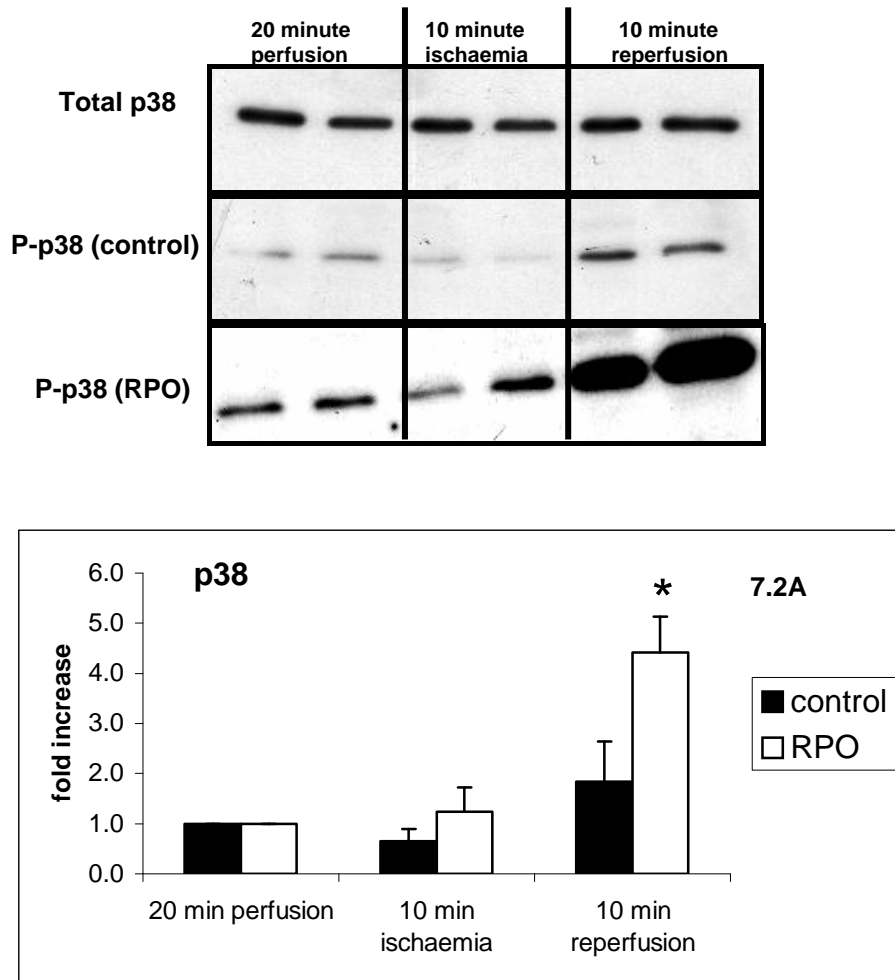


Figure 7.2A The effect of dietary RPO-supplementation on p38 phosphorylation in hearts subjected to ischaemia and reperfusion. Samples were analysed by Western blotting with phospho-specific antibodies recognizing dual phosphorylated MAPKs. Results are expressed as mean \pm SEM for 4 independent experiments ($n=4$ per group/time point). 10 minutes reperfusion RPO *versus* 10 minutes reperfusion control (* $P < 0.001$)

Ischaemia/reperfusion caused significant increases in both JNK54 and JNK46 phosphorylation of control group from 20 minutes perfusion to 10 minutes reperfusion (from $1,0 \pm 0$ fold to $3,9 \pm 0,23$ fold, $P<0,001$ for JNK54 and $1,0 \pm 0$ to $6,83 \pm 0,66$ fold, $P<0,001$ for JNK46) (Figure 7.2B).

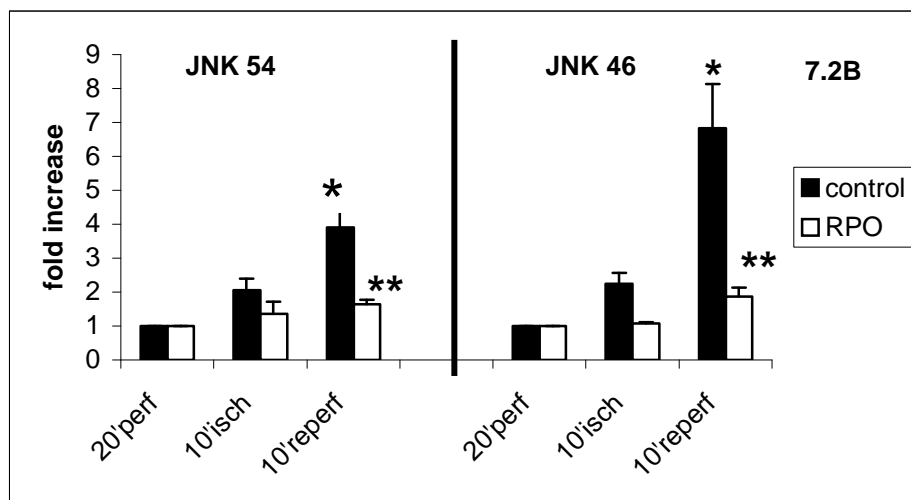
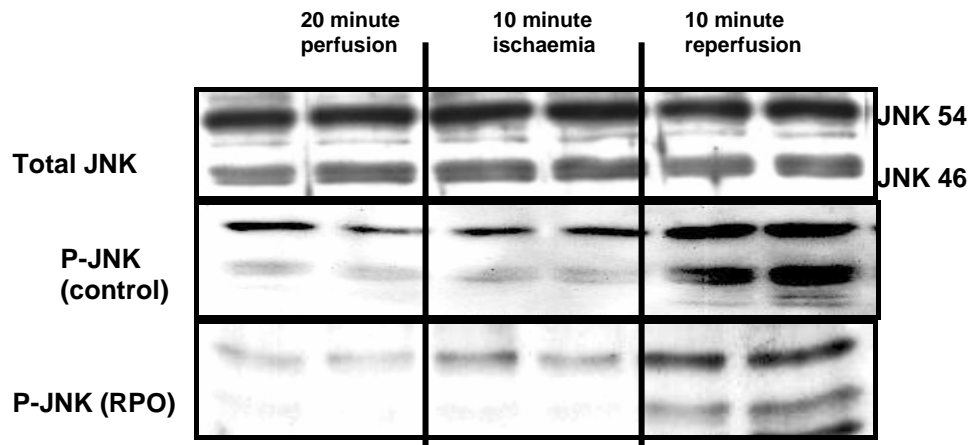


Figure 7.2B The effect of dietary RPO-supplementation on JNK phosphorylation in hearts subjected to ischaemia and reperfusion. Samples were analysed by Western blotting with phospho-specific antibodies recognizing dual phosphorylated MAPKs. Results are expressed as mean \pm SEM for 4 independent experiments ($n=4$ per group/time point). JNK54: 10 minutes reperfusion RPO *versus* 10 minutes reperfusion control (** $P<0,001$). JNK 54 and 46: 10 minutes reperfusion control *versus* 20 minutes perfusion control (* $P<0,001$); 10 minutes reperfusion RPO *versus* 10 minutes reperfusion control (** $P<0,001$)

Phosphorylation of JNK54 and JNK46 was increased significantly less in hearts of the group supplemented with RPO when compared with hearts of the corresponding control group at 10 minutes reperfusion, respectively ($1,65 \pm 0,06$ fold *versus* $3,90 \pm 0,23$ fold, $p < 0,001$ for JNK54 and $1,87 \pm 0,13$ fold *versus* $6,83 \pm 0,66$ fold, $P < 0,001$ for JNK46).

There were significant increases in ERK44 and ERK42 phosphorylation during ischaemia and reperfusion in hearts of both the RPO-supplemented and control groups when compared with baseline ERK44 and ERK42 phosphorylation. RPO-supplementation did not offer cardioprotection at any of these time points as measured by increased phosphorylation of ERK44 and ERK42 when compared with ERK44 and ERK42 phosphorylation of control groups, respectively (Figure 7.2C).

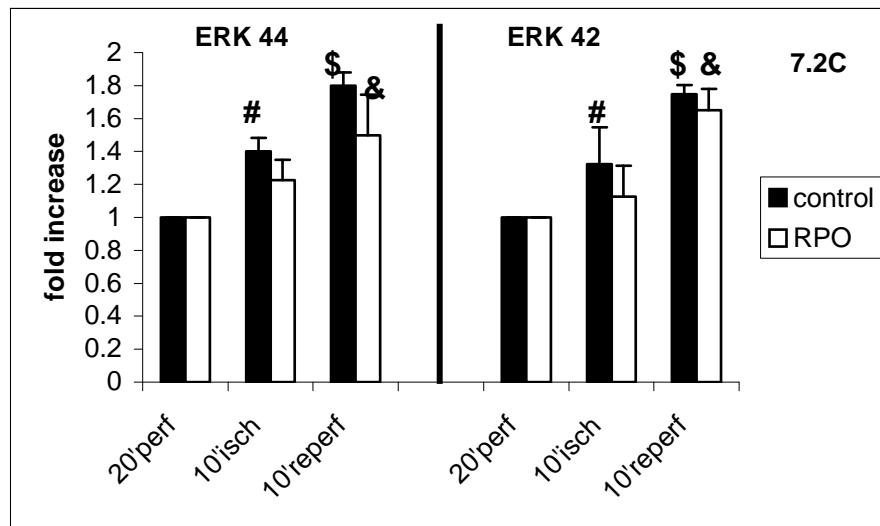
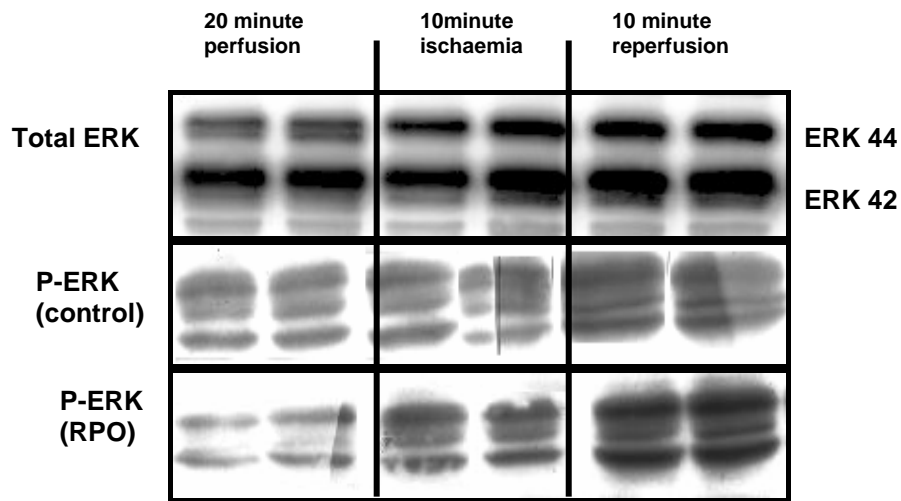


Figure 7.2C The effect of dietary RPO-supplementation on ERK phosphorylation in hearts subjected to ischaemia and reperfusion. Samples were analysed by Western blotting with phospho-specific antibodies recognizing dual phosphorylated MAPKs. Results are expressed as mean \pm SEM for 4 independent experiments (n=4 per group/time point). ERK44: 10 minutes ischaemia control *versus* 20 minutes perfusion control ([#] $P<0,01$); 10 minutes reperfusion control *versus* 10 minutes ischaemia control (^{\$} $P<0,01$); 10 minutes reperfusion RPO *versus* 20 minutes perfusion RPO ([&] $P<0,001$). ERK42: 10 minutes ischaemia control *versus* 20 minutes perfusion control ([#] $P<0,05$); 10 minutes reperfusion control *versus* 10 minutes ischaemia control (^{\$} $P<0,01$); 10 minutes reperfusion RPO *versus* 20 minutes perfusion RPO ([&] $P<0,001$)

7.4.3 The effect of RPO-supplementation on the phosphorylation of PKB/Akt in hearts subjected to ischaemia and reperfusion

Phosphorylation of PKB/Akt (Ser⁴⁷³) was determined by Western blotting using phospho-specific antibodies. There was a significant increase in PKB/Akt phosphorylation of hearts in the RPO-supplemented group compared to the control group during reperfusion (4.03 ± 1.1 fold *versus* 1.03 ± 0.11 fold, $P < 0.01$) (Figure 7.3).

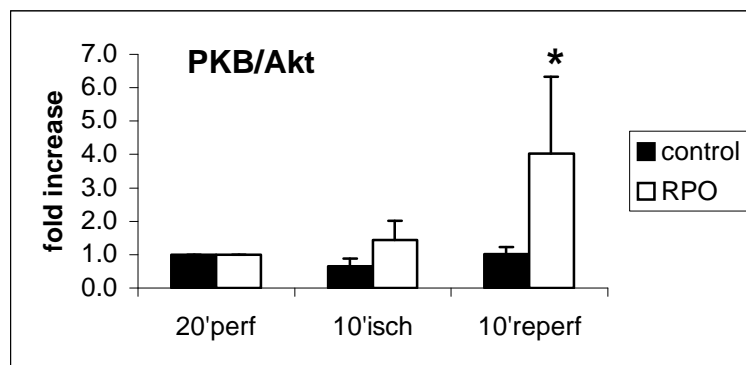
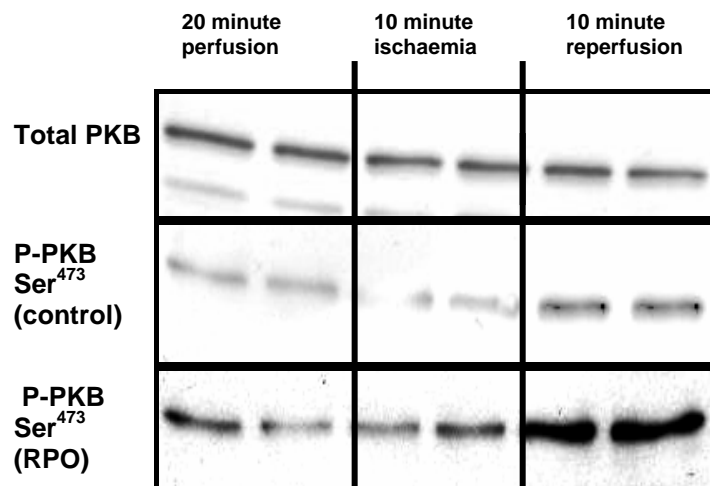


Figure 7.3 The effect of dietary RPO-supplementation on PKB phosphorylation in hearts subjected to ischaemia and reperfusion. Samples were analysed by Western blotting with phospho-specific antibodies recognizing dual phosphorylated MAPKs. Results are expressed as mean \pm SEM for 4 independent experiments (n=4 per group/time point). 10 minutes reperfusion RPO *versus* 10 minutes reperfusion control (* $P < 0.01$)

7.4.4 The effect of RPO-supplementation on PARP cleavage and caspase-3 activation in hearts subjected to ischaemia and reperfusion

The control group showed a significant increase in PARP cleavage during reperfusion compared to PARP cleavage in the control perfusion group ($2,1 \pm 0,27$ fold *versus* $1,0 \pm 0$ fold, $P<0,01$) (Figure 7.4A). However RPO-supplementation significantly attenuated PARP cleavage ($0,6 \pm 0,17$ fold for RPO-group *versus* $2,1 \pm 0,27$ fold for control group, $P<0,001$) during reperfusion.

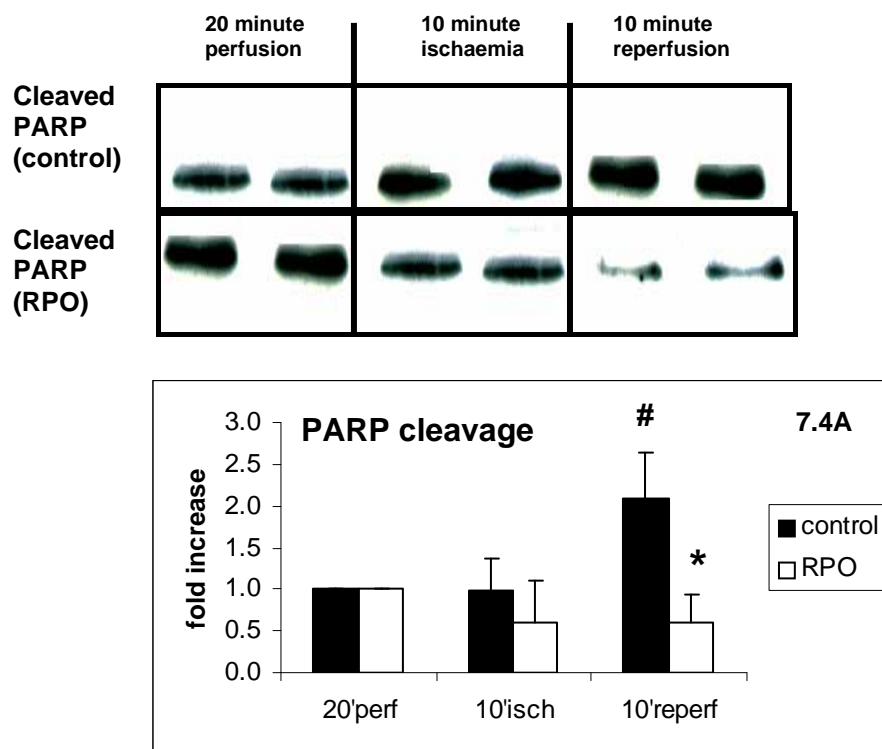


Figure 7.4A: The effect of dietary RPO-supplementation on PARP cleavage during ischaemia and reperfusion. Samples were analysed by Western blotting with antibodies recognizing cleaved PARP and caspase-3. Results are expressed as mean \pm SEM for 4 independent experiments ($n=4$ per group/time point). PARP: 10 minutes reperfusion control *versus* 20 minutes perfusion control ($^{\#}P<0,01$); 10 minutes reperfusion RPO *versus* 10 minutes reperfusion control ($^*P<0,001$).

Hearts of the control group showed a significant increase in caspase-3 activation during reperfusion when compared with caspase-3 activation of hearts during perfusion ($P<0,05$). (Figure 7.4B).

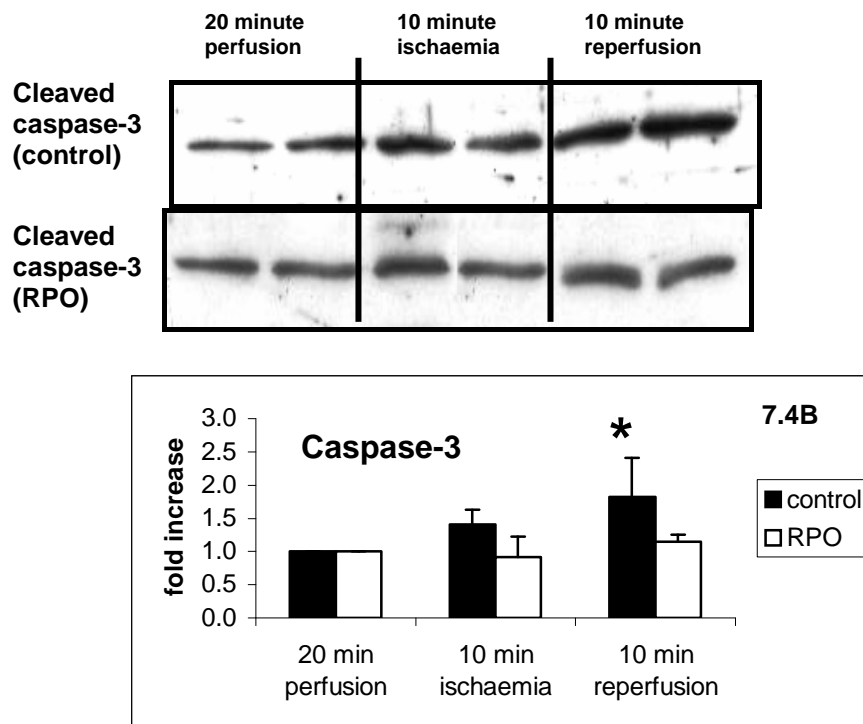


Figure 7.4B: The effect of dietary RPO supplementation on caspase-3 activation during ischaemia and reperfusion. Samples were analysed by Western blotting with antibodies recognizing cleaved PARP and caspase-3. Results are expressed as means \pm S.E.M. for 4 independent experiments (n=4 per group/per time point). Caspase-3: 10 minutes reperfusion control versus 20 minutes perfusion control (* $P<0.05$)

7.5 Discussion

We have demonstrated that RPO-supplementation offered significant protection against ischaemia/reperfusion-induced injury in the isolated perfused working heart as reflected by improved functional recovery. Except for our own, no other evidence exists for the role of RPO in functional recovery after ischaemia/reperfusion induced injury (Esterhuyse *et al.*, 2005). However,

some evidence does exist for the effect of some of the major components of RPO on cardiac function. Meehan and co-workers (1994) demonstrated that oleic acid improved functional recovery in ischaemic/reperfused hearts. Serbinova and co-workers (1992) showed that RPO vitamin E was more effective than tocopherols in protecting against ischaemia/reperfusion injury in the isolated Langendorff perfused heart. Das and co-workers (in press) also demonstrated that palm tocotrienol provided cardioprotection as proved by reduction of ischaemia/reperfused-mediated increases in ventricular dysfunction, ventricular arrhythmias and myocardial infarct size. Furthermore, Bilgin-Karabulut and co-workers (2001) has showed that pre-treatment with a combination of vitamin A and vitamin E offered protection against venous ischaemia/reperfusion-induced injury, Interestingly, these vitamins were not effective when used as single agents (Bilgin-Karabulut *et al.*, 2001).

In response to ischaemia, cells activate various signal transduction pathways which may be either harmful, or allow adaptation to this stressful environment. Recent studies suggested that the MAPKs are important regulators of apoptosis in response to myocardial ischaemia/reperfusion. Therefore, we characterized the three major MAPK subfamily members that are activated during ischaemia and reperfusion in our model and investigated the influence of RPO on their phosphorylation status.

Dietary RPO-supplementation significantly increased the generic p38 isoform phosphorylation during reperfusion (Figure 7.2A). Despite reports to the contrary (Mackay and Mochly-Rosen, 2000; Marais *et al.*, 2001), several investigators support the concept that p38 activation protects the heart from

ischaemia/reperfusion-induced injury (Maulik *et al.*, 1996; Weinbrenner *et al.*, 1997). These opposing results may be attributed to the different isoforms (α and β) expressed in the heart (Saurin *et al.*, 2000), which appear to mediate opposing effects. The p38 α -isoform is implicated in apoptosis, whereas p38 β is anti-apoptotic in rat cardiac myocytes. JNK phosphorylation (JNK54 and JNK46) was significantly increased during reperfusion but was attenuated by dietary RPO supplementation. JNK phosphorylation appears to be pro-apoptotic in many cell types (Obata *et al.*, 2000; Park *et al.*, 2000), however their exact role in regulating cell death is unclear. For example, Hreniuk and co-workers (2001) found that inhibition of JNK46, but not JNK54, significantly reduced reoxygenation-induced apoptosis. Wang and co-workers (1998), on the other hand, reported that activation of JNK by transfection of cultured rat neonatal cardiomyocytes with mitogen activated protein kinase kinase 7 (MKK7), an upstream activator of JNK, induced hypertrophy rather than apoptosis. Although dietary RPO-supplementation had no effect on ERK phosphorylation compared to the control group, the ERK cascade appears to specifically mediate cell growth and survival signals. For instance, it has been shown that inhibition of ERK enhances ischaemia/reperfusion-induced apoptosis and that sustained activation of this kinase during simulated ischaemia mediates adaptive cytoprotection in cultured neonatal cardiomyocytes (Punn *et al.*, 2000).

We also investigated the involvement of PKB/Akt in the cellular response to dietary RPO supplementation. RPO was responsible for a significant increase in PKB phosphorylation during reperfusion (Figure 7.3). This is in agreement with results of Fujio and co-workers (2000) who showed that PKB/Akt

promotes survival of cardiomyocytes *in vitro* and protects against ischaemia/reperfusion injury in the mouse heart. PKB is activated downstream of PI-3-kinase by the phosphoinositide-dependent protein kinases PDK-1 and PDK-2 (Anderson *et al.*, 1998). PKB in turn phosphorylates a number of downstream targets relevant to cell survival functions, including the pro-apoptotic Bcl-2 family member BAD (Del Peso *et al.*, 1997). Phosphorylation of BAD on Ser¹³⁶ by PKB inhibits its pro-apoptotic function, thus promoting cell survival (Datta *et al.*, 1997). Interestingly, BAD is not only a substrate for PKB, but is also phosphorylated by the MAPK kinase MEK (Punn *et al.*, 2000), linking the classical Ras-MAPK pathway to cell survival.

Apoptosis has been consistently observed in cardiac myocytes after reperfusion and may represent a direct mechanism by which myocytes are damaged (Abe *et al.*, 2000). Indeed, in our model, reperfusion injury also resulted in cleavage of PARP to its proteolyzed products, a phenomenon well known to result from caspase-3 activation. RPO-supplementation significantly reduced PARP cleavage during reperfusion, and attenuated caspase-3 activation, although not significantly.

In summary, our results have shown that RPO-supplementation caused increased phosphorylation of p38 and PKB and reduce phosphorylation of JNK. Both increased PKB and p38 phosphorylation and the inhibition of JNK phosphorylation may contribute to the protection of the cell against apoptosis. The attenuation of PARP cleavage would in turn be expected to inhibit apoptosis. Results presented in other studies (Bogoyevitch *et al.*, 1996; Punn

et al., 2000), as well as our own (Engelbrecht *et al.*, 2004), indicate that the MAPKs are central regulators of reactive signalling in cardiac myocytes. Dietary RPO-supplementation was associated with increased percentage myocardial EPA after ischaemia in our perfusion model, which may effect intracellular signalling cascades. The ability to directly manipulate MAPK signalling has been shown to protect cardiomyocytes from ischaemia/reperfusion-induced apoptosis/injury. This notion suggests that members of the MAPK signalling cascade would be ideal targets for pharmacological intervention to treat ischaemia/reperfusion injury. Therefore, according to our results, a daily dosage RPO of 0,58 mg/kg suggest to be beneficial to humans.

In the current study we have demonstrated for the first time that RPO might exert its beneficial effects during reperfusion through increased PKB/Akt and p38 phosphorylation and dephosphorylation of JNK, which might be associated with inhibition of apoptosis and improved function. Thus, RPO might offer an alternative, non-pharmacological strategy to protect the heart against ischaemia/reperfusion-induced injury.

CHAPTER 8

CONCLUSION

To our knowledge no previous studies have investigated the effect of dietary RPO-supplementation on cell signalling associated with cardioprotection during ischaemia/reperfusion injury. In the current study we provide data of dietary RPO-intervention on the signalling pathways that may be involved in cardioprotection during ischaemia and reperfusion.

An *in vitro* working rat heart perfusion model was used to investigate the effects of dietary RPO-supplementation on myocardial post-ischaemic functional recovery and the mechanisms involved. Our results clearly indicated that dietary RPO-supplementation of a standard rat chow diet protects against the consequences of global ischaemia/reperfusion in the isolated perfused rat heart as reflected by improved aortic output recovery. Based on our results, we propose that the protective effect of RPO may be associated with either its antioxidant characteristics, and/or changes in the phospholipid fatty acid composition of the myocardium during ischaemia/reperfusion injury. We hypothesize that the palm oil vitamin E antioxidant properties may contribute to elevated cGMP and decreased cAMP concentrations early in ischaemia. This may be the more prominent mechanism of cardioprotection in this model using a standard rat chow diet. It is possible that cGMP may attenuate ischaemia/reperfusion injury by inhibiting the cAMP induced increase in the slow inward calcium current, thus leading to a decrease in ischaemic cytosolic calcium concentrations.

We also investigated whether RPO-supplementation offers the same protection against ischaemia/reperfusion injury when supplemented with a

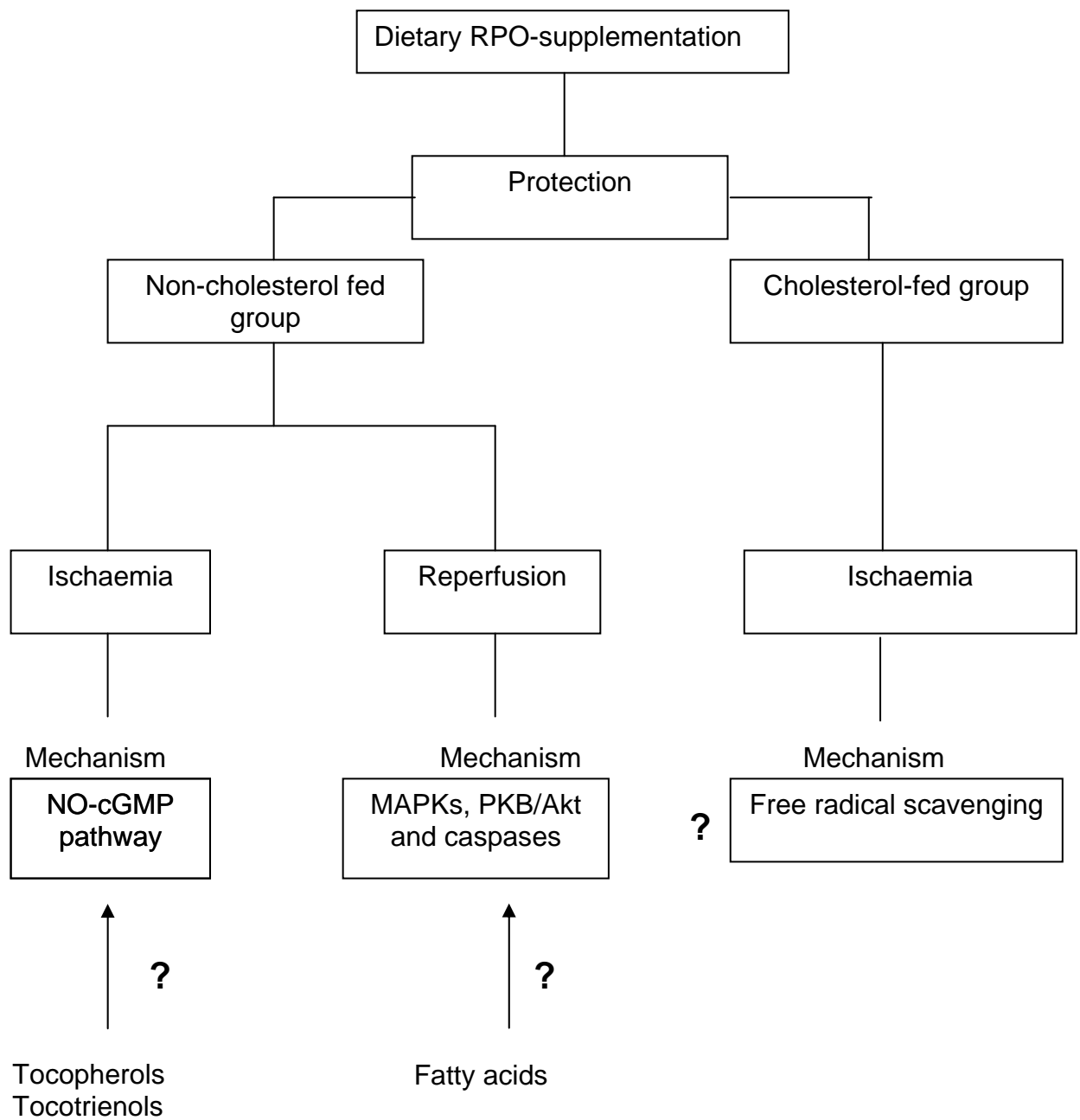
high-cholesterol diet and tried to elucidate possible mechanisms involved in this protection. Increased reperfusion aortic output recovery and rate pressure product values with RPO-supplementation of a high-cholesterol diet were associated with a decreased cAMP concentration during reperfusion. Although suppression of cAMP during reperfusion could be involved in cardioprotection, we were of the opinion that this was not the only mechanism of protection. In addition, our results clearly demonstrated that the RPO-induced protection of a high-cholesterol diet could not be linked with increased NO-cGMP signalling and confirms our hypothesis that cGMP may not be the only mechanism of protection. This following sequence of events should be considered: In the presence of cholesterol, superoxide production is increased. Superoxide would compete to re-direct the reaction towards lipid peroxidation, instead of cGMP production. Our results indicate that neither SOD activity, nor LPO production increased. We therefore speculate that RPO antioxidants acted as free radical scavengers and could potentially improve NO availability. However, we have no data to support this argument, but it does create an opportunity for future investigations.

From our work it is also evident that most of the RPO-induced changes occurred during the ischaemic period with the NO-cGMP pathway being a major role player.

Recent studies suggested that the MAPK family, PKB/Akt and signal transduction caspases are important regulators of apoptosis in response to myocardial ischaemia/reperfusion. We demonstrated for the first time that

dietary RPO-supplementation protects the isolated perfused working rat heart from ischaemia/reperfusion-induced apoptosis/injury through MAPK-, PKB/Akt- and caspase dependent pathways during the reperfusion period. Dietary RPO-supplementation exerts its beneficial effects through increased PKB/Akt and p38 phosphorylation and dephosphorylation of JNK, all of which might be associated with inhibition of apoptosis and improved functional recovery. Dietary RPO-supplementation was associated with increased percentage myocardial EPA after ischaemia in our perfusion model, which could be involved in the modulation of MAPK enzyme activity.

In summary our results suggest that dietary RPO-supplementation offered protection against ischaemia/reperfusion injury as reflected by improved aortic output recovery. We could not demonstrate whether the fatty acids (although little contribution to that of diet) or antioxidant content individually, or as a combination, was responsible for these protective effects. Our results suggest that hearts of cholesterol-fed animals were protected through a different mechanism (may possibly include the antioxidant capacity of RPO). The proposed mechanisms include RPO protection in ischaemia via the NO-cGMP pathway and MAPK, PKB/Akt and caspase involvement during reperfusion (Figure 8.1).



Note: ? Unclarified / Needs further investigations

Figure 8.1 Proposed mechanisms for dietary RPO protection

ADDENDUM

CONGRESSES

A: International

1. **International Palm Oil Congress (PIPOC 2003)**
24-28 August 2003, Putrajaya Marriott Hotel, Putrajaya , Malaysia
Paper: Dietary red palm oil supplementation improves post ischaemic functional recovery in the isolated perfused rat heart. **AJ Esterhuyse**, EF du Toit, J van Rooyen.
2. **2004 ISHR World Congress Satellite: Cellular Injury in Ischaemia, Kruger Park, South Africa, 13-15 August 2004.**
Poster: NO-cGMP pathway involved in red palm oil mediated ischaemic protection. **Jacques van Rooyen**, Johan Esterhuyse, Spinnler Benade, Eugene du Toit. (Abstract accepted and published in CVSA).
3. **Oils and fats International Congress, Putra World Trade Centre, Kuala Lumpur, Malaysia, 29 September-2 October 2004**
Invited Speaker: Red palm oil: Myth or Magic? The effect of the NO-cGMP pathway. **Van Rooyen J**; Esterhuyse AJ; Du Toit EF.

B: National

1. **32nd Annual Congress of the PSSA, September 12-15, 2004. Coffee Bay, Transkei, South Africa.**
Plenary Speaker : Red palm oil: Myth or Magic. **Jacques van Rooyen**; Johan Esterhuyse; Eugene F du Toit.

Poster: A role for dietary red palm oil induced cardioprotection in isolated rat hearts. **Esterhuyse AJ**; Du Toit EF; Bester DJ; Benadé AJS; Van Rooyen J.

Poster: Cardiac function in isolated perfused rat heart is not negatively affected by dietary red palm oil. **Bester DJ**; Van Rooyen J; Du Toit EF; Benadé AJS; Esterhuyse AJ.

2. Academic Day, University of Stellenbosch, South Africa, 18 August 2004

Poster: Dietary red palm oil offers protection against ischaemia/reperfusion injury. **Esterhuyse AJ**; Bester DJ; Van Rooyen J; Du Toit EF.

PUBLICATIONS

- 1. Esterhuyse AJ; Du Toit EF; Van Rooyen J.** Dietary red palm oil supplementation protects against the consequences of global ischaemia in the isolated perfused rat heart. **Asia Pac J Clin Nutr (accepted 2004).**
- 2. Esterhuyse AJ; Du Toit EF; Benadé AJS; Van Rooyen J.** Dietary red palm oil improves reperfusion cardiac function in the isolated perfused rat heart of animals fed a high cholesterol diet. **Prostagl, Leukot Essent Fatty Acids 2005; 72:153-161.**
- 3. Anna-Mart Engelbrecht; Johan Esterhuyse; Eugene Du Toit; Jacques van Rooyen.** p38-MAPK and PKB/AKT, possible role players in red palm oil induced protection of the isolated perfused rat heart. **J Nutr Biochem (accepted 2005).**

Submitted:

- 3. Johan Esterhuyse; Jacques van Rooyen; Hans Strijdom; Dirk Bester; Eugene du Toit.** Proposed mechanisms for red palm oil induced cardioprotection in a hyperlipidaemic perfused rat heart model. **(2005).**

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